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THE CYCLIC ADENOSINE MONOPHOSPHATE RECEPTOR PROTEIN OF ESCHERICHIA COLI : CHEMICAL AND PROTEOLYTIC MODIFICATION AS A PROBE OF STRUCTURE AND FUNCTION

by

CHRISTINE PAMPENO

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

TITLE: The Cyclic AMP Receptor Protein of Escherichia Coli: Chemical and Proteolytic Modification as a Probe of Structure and Function.

BY: Christine Pampeno

ADVISOR: Professor Joseph S. Krakow

The relationship between the structure and function of the cAMP receptor protein (CRP) of E.coli was examined by methods of chemical and proteolytic modification. The SAP-CRP fragment, produced by limited digestion of CRP (subunit M.Wt., 22,500) with Staphylococcus aureus V8 protease (SAP), has a molecular weight of 18,900 ± 500 daltons as estimated by SDS-polyacrylamide gel electrophoresis. SAP-CRP has lost DNA binding activity and the two available sulfhydryl groups of CRP while retaining the two 'buried' sulfhydryl groups and cAMP binding activity. SAP-CRP has a lower net positive charge than CRP reflecting a higher acidic to basic amino acid ratio. The products of further proteolytic digestion of SAP-CRP, using native and denaturing conditions, suggest that SAP-CRP contains the NH_2-proximal core region of CRP. The results indicate that the polypeptide segment lost following SAP digestion represents all or part of the COOH-terminal DNA binding domain of CRP.

Reaction of CRP with the bifunctional reagent, ortho-phenylenedimaleimide (oPDM), results in the crosslinking of the two subunits of a CRP protomer. In the presence of cAMP, the rate of crosslinking increases. CRP modified with oPDM retains (^{3}H)cAMP binding activity but loses (^{3}H)d(I-C)$_n$ binding activity. Proteolysis of crosslinked CRP gives distinct SDS-polyacrylamide gel electrophoretic patterns depending upon whether cAMP was present during the reaction with oPDM. CRP crosslinked in the absence of cAMP retains the same relative resistance to proteolysis as unmodified CRP. The presence of 0.1 mM cAMP during proteolysis results in the production of two fragments, one of ~13,000 daltons and a second of ~20,000 daltons. CRP crosslinked with oPDM in the presence of cAMP (then dialyzed to remove the cAMP) remains sensitive to αchymotrypsin digestion, even in the absence of added cAMP, producing only the 13,000 dalton fragment. It is suggested that the nature of the oPDM crosslink is a consequence of the conformational state of CRP.

Treatment of CRP with trinitrobenzenesulfonic acid, such that ~7 lysine residues are trinitrophenylated, inhibits the formation of cAMP-dependent and independent (^{3}H)d(A-T)$_n$ -CRP complexes. In contrast, cAMP-dependent binding of TNP-CRP to (^{3}H)d(I-C)$_n$ remains relatively unaltered. It is suggested that the differential ability of trinitrophenylated CRP to bind the two deoxypolymers may reflect the manner in which CRP interacts with the promoter site.

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ABBREVIATIONS

BTP, 2- Bis(2-hydroxyethyl)amino -2-(hydroxymethyl)-1,3-propanediol; cAMP, adenosine 3'-5'-monophosphate; cGMP, guanosine 3'-5'-monophosphate; cTuMP, tubercidin, 3'-5'-monophosphate; CRP, cAMP receptor protein; αCRP, resistant core formed by subtilisin digestion of CRP in the presence of cAMP; SAP-CRP, resistant core formed by Staph. aureus protease digestion of CRP in the presence of cAMP; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TNB, thionitrobenzoate; DTT, dithiothreitol; $d(A\text{-}T)_n$, poly(dA-dT)·poly(dA-dT); $d(I\text{-}C)_n$, poly(dI-dC)·poly(dI-dC); EDTA, (ethylenedinitrilo)tetraacetic acid; Hepes, N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MBS, meta-maleimidobenzolyl-N-hydroxysuccinimide; ME, β-mercaptoethanol; NEM, N-ethylmaleimide; oPDM, N,N'-(1,2-phenylene)bismaleimide; pPDM, N,N'-(1,4-phenylene)bismaleimide; PMSF, phenylmethanesulfonyl flouride; SAP, Staphylococcus aureus V8 protease; SDS, sodium dodecyl sulfate; TNBS, trinitrobenzenesulfonic acid ; TNP-, trinitrophenyl.

INTRODUCTION AND BACKGROUND

In prokaryotes, the initiation of transcription, at select genes, can be controlled by the interaction of 'regulatory' proteins with their cognitive sites on the DNA template. The following study examines the structure-function relationships of one such protein, the cyclic AMP receptor protein of E.coli.

An understanding of the mechanisms by which a protein recognizes and interacts with a specific DNA sequence requires knowledge of the structural nature of the protein-DNA interaction sites. In recent years, the regulatory control regions of several transcriptional 'operon' units have been sequenced, among them, the lactose operon of E.coli (Dickson et al., 1975). Two proteins regulate the initiation of transcription at the lac operon: the lac repressor acts in a negative fashion while the cAMP receptor protein (CRP) is a positive control element. As both the repressor and CRP have been isolated in adequate quantities for investigation of their structural properties, the lac operon provides a valuable in vitro model system for studying the specific interactions between DNA and proteins which regulate gene expression.

The existance of cytoplasmic 'repressor molecules' was predicted by Jacob and Monod (1961) in their operon model of gene expression, an outcome of their physiological and genetic studies of β galactosidase synthesis. The term 'operon' describes a group of genes, encoding metabolically related enzymes, whose expression is coordinately regulated by an operator-promoter locus situated at one end of the operon. The operon is expressed as a single unit of transcription into a polycistronic messenger RNA; RNA polymerase binds to the 'promoter site' such that transcription of one DNA strand occurs in the direction of the structural genes. Binding of a repressor molecule to the'operator site' prevents transcription of the operon. By reducing the affinity of the repressor for the operator, inducer molecules allow transcription to proceed. The isolation of a protein which bound the lactose operon inducer, isopropylthiogalactoside, IPTG, and had a high affinity for λ lac DNA indicated that the lac repressor was a protein molecule (Gilbert and Muller-Hill, 1967). The lac repressor has been purified to homogeneity and its amino acid sequence determined (Beyruther et al., 1975; Beyruther, 1978). By using purified repressor to isolate inducer from cells grown on lactose, Jobe and Bourgeois (1972) found allolactose to be the natural inducer of the lac operon.

The role of cAMP in the regulation of gene expression became apparent when the ability of glucose to repress the

synthesis of inducible enzymes was correlated with the discovery of Makman and Sutherland(1968) that glucose lowered cAMP levels in E.coli; the repressive effect of glucose, termed'catabolite repression', was overcome by cAMP (Perlman and Pastan, 1968; Ullman and Monod,1968). The failure of adenyl cyclase mutants to utilize lactose as well as many other carbon sources suggested that cAMP played a general role in activating the synthesis of 'catabolite repressible' enzymes (Perlman and Pastan, 1969). The specific stimulation of lac mRNA coordinately with β-galactosidase synthesis in cells growing in the presence of cAMP, IPTG and glucose suggested that cAMP acts at the level of transcription (Varmus et al., 1970). The possibility that the action of cAMP was mediated by a protein was indicated by the isolation of a class of mutants that were phenotypically similar to adenyl cyclase mutants but did not respond to exogenous cAMP (Emmer et al., 1970). Assay of E.coli cell extracts for the ability to bind (^{3}H)cAMP (Emmer et al., 1970) or for the capacity to stimulate lac DNA-directed β-galactosidase synthesis (Zubay et al., 1970), in cell free systems supplemented with cAMP (Chambers and Zubay, 1969), lead to the identification of the cAMP receptor protein (CRP) also called catabolite gene activator protein (CAP or CGA). CRP was purified to homogeneity by Anderson et al. (1971).

Controlled transcription of the lac and gal operons was achieved in vitro using purified lac or gal DNA, RNA polymerase holoenzyme, CRP-cAMP and the lac repressor (deCrombrugghe

et al.,1971; Nissley et al., 1971). Use of these systems demonstrated that both CRP and cAMP are required for the formation of rifampicin-resistant preinitiation complexes between RNA polymerase and the lac promoter. These results were in agreement with previous in vivo observations that cAMP could not stimulate β-galactosidase or lac mRNA synthesis in cells containing lac promoter mutants (Perlman et al., 1969). Specific interactions between CRP and lac DNA were indicated by studies of a fluorescent derivative of CRP which underwent a conformational change when bound to λdlac DNA but not wild type λDNA (Wu,F.Y.-H. et al., 1974). Subsequently, preferential binding of CRP-cAMP to restriction fragments containing the lac promoter was demonstrated (Majors, 1975a; Mitra et al., 1975); restriction fragments containing mutations which decrease the ability of CRP to stimulate lac gene expression showed little affinity for CRP-cAMP (Majors, 1975a). Thus, CRP-cAMP apparently functions by interacting with the promoter-related site to facilitate the initiation of transcription.

In vitro transcription studies have evoked a model for the formation of preinitiation RNA polymerase-promoter complexes (Chamberlin, 1974). Initially, RNA polymerase recognizes a specific duplex promoter sequence; this closed promoter complex is easily dissociated and is functionally inert. The closed complex undergoes a transition to form a highly stable 'open complex' from which transcription

rapidly proceeds. Formation of the open complex is favored by conditions that destabilize the double helix such as temperature, or nonionic solvents like glycerol, dimethyl sulfoxide or ethylene glycol (Nakanishi et al., 1974, 1975), suggesting that localized melting of the promoter region may be involved in the conversion from the closed to open promoter complex.

Formation of RNA polymerase promoter initiation complexes may be mediated by the sequence of the promoter region. The nucleotide sequence of the lac promoter region (Fig. A-1) reveals certain structural features which apparently lower the efficiency of preinitiation complex formation. In the lac promoter, the region corresponding to the heptanucleotide proposed by Pribnow (1975) to form a stable preinitiation complex with RNA polymerase has the sequence 5'TATGTTG 3', compared with the prototype sequence, 5' TATPuATG 3'. The lac promoter,which operates at low efficiency in the absence of CRP-cAMP, cannot form a heparin-resistant open-complex with RNA polymerase in vitro (Reznikoff, 1976). A lac promoter mutant, UV5, which increases CRP-cAMP independent lac operon transcription (Silverstone et al., 1970) and forms an in vitro open complex with polymerase (Reznikoff, 1976) has the sequence, 5'TATAATG 3', which fits the prototype heptamer and contains an A·T instead of a G·C base pair at the midpoint of the heptamer block (cited in Gilbert, 1976). The UV5 mutation may destabilize the heptamer block thereby enhancing formation of the open complex (Pastan and Adhya,

1976) or may provide better contacts for RNA polymerase. The transition from the closed to open lac promoter complex might also be inhibited by the stabilizing effect of the G·C rich blocks (Burd et al., 1975) which flank the A·T rich region proposed to be the RNA polymerase recognition and 'entry' site (Dickson et al., 1975).

The binding sites for the regulatory proteins which stimulate or inhibit transcription occur near or within the promoter region. The operator site for the lac repressor (Gilbert & Maxam, 1973; Maizels, 1973; Majors, 1975b), tryptophan repressor (Bennet et al., 1976), and λ repressors (Maniatis et al., 1975) have been shown to overlap the initiation region of the promoter. Thus, repressors can function to inhibit transcription by excluding or blocking RNA polymerase from the promoter.

The operator sites contain regions of 2-fold rotational symmetry which could be utilized in the recognition process of the repressor proteins (O'Neill, 1977). Though the symmetrical sequences can potentially form intrastrand loop structures that could serve as 'attractive' recognitory elements (Grier, 1966), it is clear, from various experimental approaches, that both lac repressor and λ repressor bind to stable double-stranded DNA (Riggs et al., 1970a; Wang,A.C. et al., 1977; Wang, J.C., 1974; Richmond and Steitz, 1976; Marians and Wu, 1976; Maniatis and Ptashne, 1973). The symmetrical axis of the DNA binding sites could serve to align the symmetrical axis of the regulatory proteins (Sobell,

1973); such a model has been proposed for the lac repressor-operator interactions (Steitz et al., 1974).

The ability of regulatory proteins to recognize double stranded DNA sequences must involve interactions with the edges of the base pairs exposed in the major and minor grooves. The helical groove contacts of the lac repressor have been investigated. Evidence suggests that the repressor predominantly contacts the minor groove of non-operator DNA (Kolchinsky et al., 1976; Richmond and Steitz, 1976; Wang, A.C., et al., 1977), while formation of the specific operator DNA complex involves contacts in both helical grooves (Ogata and Gilbert, 1977; Goedell et al., 1978). Kolchinsky et al. (1976) have proposed that the lac repressor may initially interact, nonspecifically, within the minor groove. When the proper segment is roughly recognized, a conformational change might occur which would allow interactions within the major groove. The small degree of helix unwinding which occurs when repressor binds to operator DNA (Wang, J.C. et al., 1974) may be related to such a 'two-step' lac repressor-operator interaction. Combining the accumulated observations of lac repressor-operator contacts, Goedell et al. (1978) have presented a model of lac repressor binding whereby the repressor binds to one side of the DNA helix, cutting across and interacting with both major and minor grooves. Postulated strong contacts occur with the 5-methyl group of thymine and the N^7 group of guanine in the major groove, and the 2-NH_2 group of guanine in the minor groove.

The CRP-cAMP complex, which is required for efficient utilization of the lac promoter, binds to a site located approximately 60 base pairs before the origin of transcription. This region contains 12 out of 14 base pairs that are related by 2-fold rotational symmetry (Dickson et al., 1977). Mutations in this region render the lac operon insensitive to CRP-cAMP (Miller et al., 1968; Eron and Block, 1971); restriction fragments carrying these mutations do not bind CRP (Majors, 1975a). All point mutations sequenced thus far show G·C to A·T base pair changes at one of two symmetrically related positions, suggesting that CRP makes symmetrical contacts with its binding site (Dickson et al., 1977). A putative CRP binding site has been identified in the gal operon (Musso et al., 1977a). This region is located approximately the same distance from the origin of transcription as the lac operon CRP binding site and also contains sequences of 2-fold rotational symmetry (Fig. A-1).The two binding sites show blocks of homologous sequences which may be important CRP contact points; differences between the sequences may be a consequence of the overlapping or interspersion of the gal repressor operator site with the CRP binding site (Musso et al., 1977a).

The lac promoter CRP binding site is adjacent to a G·C rich block bounding one side of the proposed A·T rich RNA polymerase entry site. Dickson et al. (1975) have suggested that the binding of CRP to its interaction site destabilizes the G·C rich region, thereby facilitating the conversion of

closed to open promoter complexes. This prediction is supported by the ability of DNA-denaturing reagents to replace the requirement of CRP-cAMP for gal mRNA synthesis (Nakanishi et al., 1974) and the ability of CRP-cAMP to lower the transition temperature for the formation of lac and gal promoter preinitiation complexes (Nakanishi et al., 1975). The possibility that CRP-cAMP stimulates transcription by direct interaction with RNA polymerase should also be considered, particularly in light of evidence which demonstrates that polymerase simultaneously contacts the initiation site as well as a region 30-40 base pairs upstream from the initiation site of the lac operon (Johnsrud, 1978).

The effect of the CRP-cAMP complex may be dictated, in part, by the individual promoter structures of the various inducible operons. Though maximal expression of the gal operon requires CRP-cAMP (Miller et al., 1971), a CRP-cAMP independent mode of gal expression also exists (Musso et al., 1977b). The gal regulatory region lacks the G·C rich blocks present in the lac promoter (Musso et al., 1977a) and has been found to contain a CRP-independent promoter which precedes the CRP-dependent promoter by 5 base pairs (Musso et al., 1977b). The low G·C content of the gal promoter may decrease the requirement of CRP-cAMP for preinitiation complex formation at the CRP-independent promoter. When CRP-cAMP binds at the gal operon, transcription is stimulated from the CRP-dependent gal promoter and repressed at the CRP-independent gal promoter (Musso et al., 1977b). Thus, CRP-cAMP exerts

both positive and negative control on gal operon transcription. The ability of CRP-cAMP to function as a negative effector has also been suggested by observations that CRP-cAMP depresses the in vivo synthesis of enzymes involved in glutamine (Prusiner et al., 1972) and deoxynucleoside (Hammer-Jeperson and Nygaard, 1976) metabolism. Details of CRP-cAMP action will be better understood when sequences and protein binding sites have been determined for other operons under cAMP control. The arabinose operon would be of particular interest since higher levels of cAMP are required for induction compared with lac operon expression (Lis and Schleif, 1973).

The DNA binding properties of CRP and the lac repressor are mediated by their respective effector molecules, cAMP and IPTG, which 'signal' environmental changes. Thus, both CRP and the repressor are proteins with two functional sites: one site interacts with DNA, the other site binds a small ligand molecule. Characterization of the structure and function of each site and their inter-relationship is required to understand the mechanism of either protein's action.

The lac repressor is a tetramer of identical subunits of molecular weight, 38,000 (Beyruther et al., 1975). Repressor binds tightly to operator DNA with a dissociation constant in the order of 10^{-13} M (Riggs et al., 1970b). Binding to non-operator DNA occurs with a dissociation constant in the order of 10^{-10} M (Lin and Riggs, 1972). The appreciable affinity of repressor for non-operator DNA has been proposed

to play an integral role in the overall regulatory mechanism by controlling the concentration of free repressor in the cell (Kao-Huang et al., 1977; Linn and Riggs, 1975a) and by accelerating the interaction of repressor with operator DNA (Lin and Riggs, 1975a; Wang,A.C. et al., 1977). While binding of the inducer, IPTG, does not affect the affinity of repressor for non-operator DNA (Lin and Riggs, 1975b), IPTG decreases the affinity of repressor for operator DNA by 1000-fold (Barkley et al., 1975). Loss of operator binding function presumably results from the conformational changes produced throughout the repressor molecule upon IPTG binding (Laiken et al., 1972; Friedman et al., 1976; Wu,C.-W., et al., 1976).

Genetic analysis of lac repressor mutants indicates that operator binding involves the NH_2-terminal region of the repressor, whereas interactions with the inducer involve regions clustered near the center of the repressor subunit (Pfahl et al., 1974). The location of distinct inducer and DNA binding functional sites is corroborated by the biochemical characterization of a repressor nonsense mutant from which 42 NH_2-terminal amino acids are missing (Platt et al., 1972) and of 'core proteins' derived from trypsin or chymotrypsin digestion of native repressor (Platt et al., 1973; Files and Weber, 1975). Proteolytic cores missing 60 NH_2-terminal and 20 COOH-terminal residues have been isolated as well as cores devoid of only the NH_2 -terminal fragment. The nonsense mutants and the core proteins cannot bind DNA

but show normal IPTG binding and tetramer formation. These observations argue that inducer and DNA binding sites are spacially separate. Further evidence also suggests that the two binding domains represent independently stabilized conformations. The proteolytic cores maintain much of the native repressor structure (Huston et al., 1974) and are able to undergo conformational change upon IPTG binding (Matthews, 1974). The NH_2-terminus is not required for renaturation, from 6 M guanidine-HCl, of tetrameric core proteins with restored IPTG binding function (Platt et al., 1973; Files and Weber, 1975). The NH_2-terminal 'headpieces' have also been isolated (Geisler and Weber, 1977). These NH_2-terminal fragments, which contain the first 50 or 60 amino acid residues of the repressor and possess secondary structure shown by circular dichroism (Jovin et al., 1977), exist in monomeric form. While filter binding assays (Geisler and Weber, 1977; Jovin et al., 1977) indicate that the headpieces are capable of only weak non-operator DNA interactions, methylation-protection experiments (Ogata and Gilbert, 1978) demonstrate that the headpieces can bind specifically to the lac operator.

The structure of the lac repressor has been predicted from its amino acid sequence (Chou et al., 1975; Patel, 1975). The proteolytic core region is characterized by extreme hydrophobicity and extensive β sheet structure. The NH_2-terminal region consists of β sheet and α helical segments which are able to form the right-hand twisted, anti-parallel, β-pleated sheet (β ribbon) structure that has been proposed to

structurally complement and recognize double stranded DNA (Church et al., 1977; Seeman et al., 1976). The amino terminus contains 50% of the tyrosine residues present in a repressor subunit. Chemical modification of these tyrosine residues has been shown to decrease the affinity of the repressor for operator DNA (Fanning, 1975; Alexander et al., 1977); tyrosine may participate in DNA binding by intercalating between base pairs or by H-bond formation (Helene, 1971). The H-bonding potential of serine and threonine residues, present in the NH_2-terminal region, would also contribute to repressor DNA interactions. Sequence analysis of the NH_2-terminal regions of several operator mutants reveal substitutions for serine or threonine (Weber et al., 1972) which would maintain the predicted secondary structure of the DNA binding region (Patel, 1975) but which cannot form H-bonds. The NH_2-terminus also contains several basic amino acids which can account for the electrostatic interactions between repressor and DNA (Riggs et al., 1970a). The COOH-terminus of the lac repressor shares several features with the NH_2-terminal region: a short β sheet structure, a relatively high proportion of positively charged amino acids and several serine and threonine residues. Thus, Chou et al. (1975) have proposed that the COOH-terminus of the lac repressor, which shows an interesting homology with the COOH-terminus of histone IV, may also be involved in some component of operator binding function.

CRP has a molecular weight of 45,000, consists of two identical subunits and is a basic protein with a pI of 9.12. (Anderson et al., 1971). A CRP protomer apparently binds one cAMP molecule with a dissociation constant of 1×10^{-5} M (Anderson et al., 1971). Acting as an allosteric effector, cAMP alters the structure of CRP to enhance its affinity for DNA. Evidence indicating that cAMP induces a conformational change in CRP includes the ability of cAMP to alter the properties of fluorescent probes covalently attached to CRP (Wu et al., 1974) and the differential susceptibility of CRP to proteolysis in the presence and absence of cAMP (Krakow and Pastan, 1973). Studies with cAMP analogues (Anderson et al., 1972; Krakow, 1975) indicate that the cAMP binding site of CRP interacts with the purine ring, the 2'-hydroxyl of the ribosyl group and the cyclophosphate group of cAMP. The N^6-amino group of adenine is necessary for induction of conformational change in CRP required for DNA binding activity (Krakow, 1975).

The relationship between CRP structure and function has been examined by methods of chemical and proteolytic modification. Digestion of CRP with various proteolytic enzymes in the presence of cAMP results in the production of resistant cores which retain the ability to bind cAMP but no longer bind DNA (Krakow and Pastan, 1973; Eilen et al., 1978). The susceptibility of the DNA-binding region and the resistance of the ligand-binding region to proteolytic attack bears a striking similarity to the effect of proteolysis on

the lac repressor (Platt et al., 1973). In contrast, however, the degradation of the DNA binding domain of the lac repressor occurs irrespective of the presence of IPTG while digestion of the DNA-binding region of CRP is dependent upon the presence of cAMP. The accessibility of their DNA binding domains to proteolytic attack reflects the biological properties of the two proteins (Krakow and Pastan, 1973); CRP requires cAMP to bind DNA while the repressor binds operator DNA with high affinity in the absence of IPTG, and non-operator DNA with lower affinity irrespective of IPTG.

The αcore (αCRP), produced by subtilisin digestion in the presence of cAMP, has been characterized by Eilen and Krakow (1977a&b; Eilen et al., 1978). The αCRP has a molecular weight of 25,000 and retains the dimeric strucure and globular shape of CRP (Eilen et.al., 1978). The 12,500 dalton αCRP subunit contains the same NH_2-terminal sequence as the native, 22,500 dalton, CRP subunit (Schlesinger, 1978). A CRP subunit contains two cysteinyl residues only one of which is readily titratable by sulfhydryl reagents (Anderson et al., 1971); the αCRP subunit retains only the 'buried' cysteinyl groups (Eilen et al., 1978). The αCRP studies have defined two structurally distinct regions of the native CRP protein: the NH_2-proximal αcore region containing two buried sulfhydryl groups, subunit interaction sites and the cAMP binding domain, and the COOH-terminal β region containing two available sulfhydryl groups and the DNA binding

domain.

The cysteinyl residues which serve as markers for the α and β regions of CRP have also been important indicators of conformational transitions. Eilen and Krakow (1977a) have demonstrated that cAMP can stabilize or 'tighten' the αCRP core to prevent exposure of the buried cysteines to sulfhydryl reagents. This 'tightening' of the α core region has been postulated to be a conformational signal which effects the formation of the DNA binding site of CRP (Eilen and Krakow, 1977a). The two available cysteinyl residues of the CRP protomer are involved in some component of DNA binding activity. Titration of these groups with dithiobis-(nitrobenzoic acid) (DTNB) results in the loss of cAMP-dependent DNA binding activity which is regenerated by displacement of the TNB group with mercaptoethanol (Eilen and Krakow, 1977a). The conformational change induced by cAMP has been shown to alter the position of the available cysteinyl residues. Fluorescent probes, covalently linked to the sulfhydryl groups, show changes in their emission spectra in the presence of cAMP, interpreted as shifts in their microenvironment (Wu,F. Y.-H., et al., 1974). Eilen and Krakow (1977b) have shown that cAMP stimulates the formation of a DTNB mediated disulfide bond crosslinking the two subunits of a CRP protomer.

The present study extends the investigations of CRP structure-function relationships. Incubation of the CRP-

cAMP complex with Staphylococcus aureus V8 protease (SAP), which cleaves at the COOH side of glutamyl and aspartyl residues (Houmard and Drapeau, 1972), has been shown to form a CRP core composed of two 18,000 ± 500 dalton polypeptides. This larger SAP-CRP core was characterized relative to CRP and αCRP to further resolve regions of native CRP function. To study the relationship between the two available cysteinyl groups as a probe for conformational changes in the β region of CRP, the bifunctional reagent ortho-phenylene-dimaleimide (oPDM) has been employed in the presence and absence of cAMP. The role of lysine residues in the binding of CRP to DNA was investigated by modification of CRP with trinitrobenzenesulfonic acid.

MATERIALS AND METHODS

1. Source of Materials.

A) General reagents and materials. All biochemicals were reagent grade. BTP, Hepes, lysozyme, ME, Sephadex G-100, Tris-OH, α chymotrypsinogen, insulin and ovalbumin were from Sigma Chemical Co., St. Louis,Mo. Brij 58, glycine and PMSF were products of Pierce Chemical Co., Rockford, Ill. Bio Rex 70, Cellex N-1 and SDS were obtained from Bio-Rad Labs., Richmond, CA. EGME was from Baker Chemical Co., Phillipsburg, NJ and Liquifluor was from New England Nuclear, Boston, MA. Acrylamide and toluene were from Eastman Chemicals, Rochester, NY and bis-acrylamide and polyethyleneimine (50%) were from Gallard-Schlessinger Chemical Co., Carle Place, NY. Ammonium sulfate was obtained from Research Plus, Denvill, NJ and EDTA was from Mallinckrodt, St. Louis, Mo. Nitrocellulose filters (0.45 μm pore size) were products of Matheson-Higgins, Woburn, MA. GF/C glass fiber filters and DEAE cellulose (DE-52) were from Whatman, England. Ultra pure urea , bovine serum albumin, horse heart cytochrome c and sperm whale myoglobin were products of Schwarz/Mann, Orangeburg, NY. Calf thymus DNA was a product of Worthington, Freehold, NJ. All reactions were performed in polystyrene tubes obtained form Starstedt, Princeton, NJ.

B) Proteolytic Enzymes. αchymotrypsin (37 U/mg), subtilisin BPN' (8.5 U/mg) and trypsin (8575 U/mg) were products of Sigma Chemical Co. Staph. aureus V8 protease (660 U/mg) was obtained from Miles Labs., Elkhart, Ind,

C) Nucleotides and polydeoxynucleotides. cAMP, cGMP and 5'AMP were from Sigma Chemical Co. cTuMP was a gift from Dr. Ira Pastan. $d(I\text{-}C)_n$ and $d(A\text{-}T)_n$ were from P-L Biochemicals, Milwaukee, Wis.

D) Radioisotope labeled reagents. (^{3}H)cAMP and (^{14}C)NEM were form New England Nuclear. (^{3}H)$d(I\text{-}C)_n$ and (^{3}H)$d(A\text{-}T)_n$ were prepared with E.coli DNA polymerase (Jovin et al., 1969) using (^{3}H)dCTP or (^{3}H)TTP obtained from New England Nuclear.

E) Chemical modification reagents. DTNB, MBS and TNBS were purchased from Pierce Chemical Co. oPDM and pPDM were obtained from Aldrich, Milwaukee, Wis.

F) Bacteria. CRP was isolated from E.coli KLF 41/JC 1533 which is diploid in the CRP structural gene. The cells were generously provided by Dr. Ira Pastan.

G) Core fragment of CRP. The αCRP was prepared by Eric Eilen by subtilisin digestion of CRP in the presence of cAMP followed by chromatography on DNA-cellulose and Bio-Rex 70 (Eilen and Krakow, 1977a).

2. Solutions.

A) Buffers. CRP purification buffers include: buffer A, 50 mM potassium phosphate (pH 6.8) and 0.1 mM EDTA; buffer B, 10 mM potassium phosphate (pH7), 0.1 mM EDTA and 0.1 M KCl; buffer C, 20 mM potassium phosphate (pH 6.5 or pH 8), 1 mM EDTA and 0.1 M NaCl. Stock solutions of 0.2 M Hepes (pH 8) and 0.5 M sodium phosphate (pH 7) were used to prepare various reaction mixtures. All buffer solutions were

prepared with deionized water and stored at 4°C; when required, pH adjustments were made with either HCl or NaOH.

B) Proteolytic enzyme solutions. All stock protease solutions (0.5 to 1 mg/ml) were prepared at 4°C in 0.1 M sodium phosphate (pH 7) directly before use.

3. Preparation of Chromatographic materials.

A) Bio Rex 70. The cation exchange resin was suspended overnight in an equal volume of 1 M K_2HPO_4 followed by extensive washing with deionized water. The Bio Rex-70 suspension (50%, v/v) was adjusted to pH 7 with HCl. After 15 minutes, the supernatant was decanted and the resin resuspended in an equal volume of buffer A.

B) Sephadex G-100 and Bio-Gel P-10. The beads were allowed to swell overnight in the equilibrium buffers described in the text.

C) DEAE-cellulose. Preswollen DEAE-cellulose (DE-52) was equilibrated with 50 mM Tris-HCl (pH 8.2) and 0.1 mM EDTA.

D) Denatured DNA-cellulose. DNA cellulose was prepared by the method of Alberts and Herrick (1971) except that the calf thymus DNA solution (2 mg/ml in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA) was heated to 95°C prior to rapid mixing with Cellex N-1 cellulose at -15°C in a salted ice bath. The preparations contained about 0.5 mg of DNA per ml of packed volume.

4. Purification of CRP.

Standardization of the purification procedure was

performed in collaboration with Eric Eilen (Eilen et al., 1978). Unless otherwise indicated, all steps were carried out at 4°C.

Cell lysis: Frozen E. coli cells (500 g) were suspended in 1.5 L of 75 mM Tris-OH by stirring with a Waring blender. After warming to 20°C, the suspension was rapidly adjusted to pH 8 with KOH and 50 ml of 0.5 M EDTA (pH 7.6) was added. Lysozyme was added (300 mg in 50 ml of 20 mM Tris-HCl, pH 8) and the mixture was stirred for 20 minutes. After cooling the lysate to 12°C, the following were added: 50 ml of 1 M Tris-HCl (pH 8), 90 ml of 0.5 M $MgSO_4$, and 50 ml of Brij 58. After stirring for 10 minutes, the viscous lysate was dispersed in 400 ml batches by sonication for one minute at 100 watts in a Model W 185 Sonicator Cell Disrupter (Heat System-Ultrosonics Inc., Plainview, NY). More recently, a Tekmar Super Dispax SD 45 has been employed to disperse the cell lysate (Eilen et al., 1978). The dispersed lysate was centrifuged at 12,000 rpm for 20 minutes in the JA-14 rotor of the Beckman J21 centrifuge. The supernatant was readjusted to pH 8.

Polyethyleneimine titration: Polyethyleneimine precipitation of nucleic acids and associated proteins was performed at pH 8 where CRP is dissociated from DNA. Supernatant samples (2 ml) were titrated with 5% polyethyleneimine (w/v, pH 8) to determine the maximum amount of polyethyleneimine which would not result in the loss of CRP as assayed by cAMP binding activity. Generally, about 0.1 ml of 5%

polyethyleneimine per ml supernatant was added dropwise to the extract, with stirring, over a period of 20 to 30 minutes. After centrifugation at 12,000 rpm for 20 minutes, the supernatant was adjusted to pH 7 with 1 M acetic acid.

Bio Rex 70 Chromatography: 300 ml of Bio Rex 70 suspension was added to the polyethyleneimine supernatant. After stirring for 10 minutes, the beads were allowed to settle (~20 minutes) and the supernatant was assayed for (^{3}H)cAMP binding activity. Generally, less than 15% of the activity remained in the supernatant, which was discarded. The Bio Rex beads were batch washed with 2 L volumes of buffer A until the absorbance at 280 nm fell below 0.1. The resin was then poured into a glass column to form a bed of approximately 3 x 25 cm. Protein was eluted with a linear 0 to 1 M KCl gradient (1L total volume) in buffer A; 20 ml fractions were collected. The cAMP binding activity elutes between 0.25 and 0.32 M KCl. Pooled fractions were concentrated by adding ammonium sulfate to 60% saturation at pH 6.8-7.0. After centrifugation at 12,000 rpm in the Beckman JA-20 rotor for 20 minutes, the precipitate was redissolved in 5 to 10 ml of buffer B.

Sephadex G-100 Chromatography: The concentrated Bio Rex fraction was loaded onto a 3 x 100 cm Sephadex G-100 column equilibrated with buffer B. The protein was eluted in 10 ml fractions at 30 ml/hr, and fractions containing (^{3}H)cAMP binding activity were pooled. Recently, better resolution of CRP has been attained with Sephacryl S-200 beads (Eilen et al., 1978).

DNA-cellulose Chromatography: The Sephadex pool, adjusted to pH 6.5 and a conductivity of 3 mmho, was loaded onto a 1.8 x 10 cm column of denatured DNA celluose previously equilibrated at pH 6.5 with buffer C. The column was washed with 50 ml of buffer C (pH 6.5) followed by elution of CRP with buffer C at pH 8; 10 ml fractions were collected. The column was stripped with 2 M NaCl in buffer C to remove other proteins which bind DNA but not cAMP; these fractions were discarded.

Bio Rex 70 concentration: The pooled DNA-cellulose fractions were loaded onto a 0.7 x 14 cm Bio Rex 70 column equilibrated with buffer A containing 0.1 M NaCl. The protein was eluted (2 ml fractions) in a salt step with buffer A containing 1 M NaCl. Peak fractions were pooled and stored at -20°C.

A summary of the purification steps is presented in Table A-1. This method of purification results in a 60% recovery of cAMP binding activity. Approximately 30 mg of CRP is obtained from 500g of frozen E.coli cells. The CRP, which is about 800-fold purified generally has a specific activity of 6000 units/mg (1 unit binds 1 pmole (^{3}H)cAMP; Anderson et al.,1971) although this value can range from 4000 to 7000 U/mg depending upon the batch of E.coli cells used. For a given batch of E.coli cells, however, the procedure is very reproducible. The purified CRP migrates as a single band on SDS-polyacrylamide gels (Eilen et al., 1978).

5. Preparation of SAP-CRP.

The SAP digestion mixture contained (final volume 37.5 ml) 15 mg CRP, 1 mg SAP, 0.1 mM cAMP, 10 mM DTT, 10 mM sodium phosphate (pH 8), 1 mM EDTA and 0.1 M NaCl. The approximate incubation time was 40 minutes at 37°C. The exact time of digestion was ascertained for each SAP lot by running a scaled-down time course and visualizing the products on SDS-polyacrylamide gels. This procedure is required since the SAP fragment is eventually degraded to a 13000 dalton limit core. PMSF was added to a final concentration of 0.1 mM to terminate the reaction. To separate CRP from protease the sample was applied, at pH 8, to a 0.7 x 5 cm DEAE column and washed with 50 mM Tris-HCl, pH 8.2, and 0.1 mM EDTA; CRP is not retained while SAP is bound by the DEAE cellulose. SAP-CRP was resolved from undigested CRP on a Bio Rex 70 column (0.7 x 10 cm) equilibrated with 20 mM potassium phosphate (pH 7) and 0.1 mM EDTA. The protein was eluted with a linear 0 to 0.5 M NaCl gradient (200ml total volume) in the equilibration buffer. Fractions containing (^{3}H)cAMP binding activity were analyzed on 12.5% SDS-polyacrylamide gels. The results show that SAP-CRP elutes between 0.2 and 0.26 M NaCl while CRP elutes between 0.32 and 0.37 M NaCl. Fractions containing SAP-CRP were pooled and concentrated on a Bio Rex column as described in the CRP purification procedure.

6. Preparation of oPDM Crosslinked CRP.

Stock solutions of 4 mM oPDM were made in acetone. When required, dilutions were made in 0.1 M Hepes (pH 8) just

prior to use. Unless otherwise stated, crosslinked samples were prepared by addition of a 10 fold molar excess of oPDM to CRP in reaction mixtures containing 0.1 M Hepes (pH 8) and 0.1 mM cAMP where indicated. The CRP concentration was routinely 1 mg per ml. After incubation at 37°C for 90 minutes, DTT was added to a final concentration of 40 mM. Samples of 1 to 2 ml were dialyzed at 4°C for 5 hours vs 1 L of 10 mM Tris-HCl (pH 8), 1 mM EDTA and 0.5 M NaCl, then overnight vs 2 L of 10 mM Tris-HCl (pH 8), 1 mM EDTA and 0.1 M NaCl. Approximately 10% of the protein precipitated out of solution. Samples were therefore centrifuged at 10,000 rpm for 20 minutes in a Beckman J-21 rotor and the supernatant protein used. Under these conditions 95-100% of the protein was crosslinked as determined from densitometry tracings following electrophoresis on 12% SDS-polyacrylamide gels. Control samples were given the same treatment as oPDM-CRP.

7. Preparation of MBS Crosslinked CRP.

Stock solutions of 5 mM MBS were made in acetone. Reaction mixtures contained per ml: 1 mg CRP (22 nmoles), 220 nmoles MBS, 50 mM sodium phosphate (pH 7) and 1 mM cAMP where indicated. After incubation at 37°C for 60 minutes, DTT was added to a final concentration of 40 mM. Samples were dialyzed overnight vs 2 L of 20 mM Tris-HCl (pH 7) and 0.1 M NaCl. Precipitated protein was removed by centrifugation. Control samples were carried through the same treatment as MBS modified CRP.

8. Preparation of Trinitrophenylated CRP.

Stock solutions of 10 mM TNBS were made in 50 mM sodium phosphate, pH 7. Reaction mixtures contained (final volume 1 ml): 1 mg CRP (22 nmoles), 2500 nmoles TNBS and 50 mM Hepes (pH 8). After incubation for 5 minutes at 25°C, reactions were stopped by addition of DTT to 10 mM. Samples were applied to a Bio Gel P-10 column (1 x 12 cm), equilibrated with 50 mM Hepes (pH 8), 0.1 mM EDTA, 0.1 M NaCl and 10 mM DTT. Fractions of 0.5 ml were collected and assayed for protein content. Both unmodified and TNP-CRP eluted in the void volume; protein recovery was 70%. To determine the extent of modification, the protein was subjected to dialysis overnight against 50 mM potassium phosphate (pH 7), 0.1 M NaCl and 0.1 M EDTA at 4°C. The number of TNP-groups per CRP protomer were determined from the absorbance at 367 nm ($\varepsilon = 1.05 \times 10^4\ M^{-1}cm^{-1}$, Goldfarb, 1966). The protein sample was read against a blank which contained the reaction mixture minus CRP and which was carried through the same procedure.

9. (^{3}H)cAMP Binding Assay.

The procedure was essentially that of Anderson et al. (1971). Assay mixtures contained (final volume 0.1 ml): 10 mM potassium phosphate (pH 7.5), 10 mM 5'AMP, 10^{-6} M (^{3}H)cAMP (550 cpm per pmole) and aliquots of CRP up to 100 ul. After incubating for 2 minutes at 37°C, 0.5 ml of saturated ammonium sulfate (pH 7.6) was added. Casein, 200 ug, was

added as a carrier protein when purified CRP was assayed. The precipitates were collected on Whatman GF/C glass fiber filters and washed with 2 ml of 60% saturated ammonium sulfate (pH 7.6). The filters were extracted with 10 ml of a solution of 3:5 parts of ethylene glycol monomethyl ether (EGME) to Liquifluor-toluene and counted in a Beckman LS-230 liquid scintillation counter. All values were corrected by subtracting counts observed in control samples which contained 1 mM nonradioactive cAMP in addition to (^{3}H)cAMP. One unit of cAMP binding activity is defined as that amount required to bind 1 pmole of cAMP (Anderson et al., 1971).

10. (^{3}H)d(I-C)$_n$ and (^{3}H)d(A-T)$_n$ Binding Assays.

The procedure was that of Krakow and Pastan (1973). Assay mixtures contained (final volume 0.25 ml): 40 mM BTP (pH 8), 2 ug CRP, 3.5 nmoles (^{3}H)deoxypolymer and 0.4 mM cAMP when indicated. Mixtures were incubated 5 minutes at 37°C. After addition of 0.75 ml of 50 mM NaCl, the mixtures were filtered onto nitrocellulose membranes which were then dried and counted in Liquifluor-toluene. All values were corrected for counts observed in the absence of CRP. Prior to use the nitrocellulose filters were soaked 30 minutes in 0.1 M KOH, thoroughly washed with deionized water and stored in buffer containing 20 mM Tris-HCl (pH 7.8) and 50 mM NaCl.

11. Protein Determinations.

Protein was determined by the method of Schaffner and

Weissmann (1973) using bovine serum albumin as a standard.

12. Determination of cAMP dissociation Constants and Number of Binding sites.

Equilibrium binding of (^{3}H)cAMP was performed using an ultrafiltration method described by Paulus (1969). Binding assays contained (final volume 0.25 ml): 10 ug CRP, 40 mM BTP (pH 8) and (^{3}H)cAMP (530 cpm per pmole) in concentrations ranging between 10^{-6} and 10^{-4} M. The ultrafiltration cell and PM-10 membranes were obtained from Medical Research Apparatus Corp., Boston, MA. The data was analyzed according to the method of Scatchard (1949). Representative Scatchard plots are shown in Figure A-2.

13. SDS-Polyacrylamide Gel Electrophoresis.

Electrophoresis on SDS-polyacrylamide gels was performed according to the method of Laemmli (1970). Slab gels were run at 30 mamps in an apparatus purchased from the Aquaboge Machine Shop, Aquaboge, NY. Running gel dimensions were 0.15 cm x 12.5 cm x 15.5 cm; a 1 cm spacer was used when required. Cylindrical gels (0.5 x 12.5 cm) were run at 5 ma/tube in an apparatus of the Hoefer Co., San Francisco, CA. Both types of gels were run until the tracking dye (0.001% bromphenol blue) reached approximately 1 cm from the bottom of the gel. The cylindrical gels were removed from the tubes with a Bio-phore gel tube eliminator (Bio-Rad Labs., Richmond,CA). Slab gels and cylindrical gels were stained 30 and 60 minutes, repectively, at 60°C with

0.2% Coomassie Blue made up in the destaining solution of 25% isopropyl alcohol, 10% acetic acid. The densitometer on the Beckman Acta III spectrophotometer was used to scan strips cut from the slab gels. Protein peaks were quantitated with a Hruden planimeter. Correlation between peak area and amount of protein applied to the gel revealed a linear relationship between 2 to 20 ug.

14. Conductivity Measurements.

Conductivity was measured on a CDM 2d conductivity meter (Radiometer Co., Copenhagen, Denmark). Salt concentrations were determined from standard curves relating conductivity to salt molarity in the appropriate experimental buffers.

RESULTS

1. Characterization of the SAP-CRP Core.

A) Production of the SAP-CRP core.

Incubation of CRP with the Staph. aureus V8 protease (SAP) in the presence of cAMP produces a polypeptide estimated to be 18,000 ± 500 daltons by SDS-polyacrylamide gel electrophoresis (Figs. 1 and 2). CRP remains relatively resistant to SAP, at protease concentrations as high as 26.6% (w/w) relative to CRP, in the absence of cAMP or when cGMP is substituted for cAMP (Fig. 1). The differential susceptibility of CRP to SAP digestion in the presence and absence of cAMP follows the pattern observed when CRP is digested with subtilisin, chymotrypsin, or trypsin (Fig. 2 and Krakow and Pastan, 1973; Eilen et al., 1978). The molecular weights for the core polypeptides formed after digestion by subtilisin and trypsin in the presence of cAMP were ∿12,500, and after chymotrypsin digestion, ∿13,000.

B) Activity Studies.

While SAP-CRP retains the cAMP binding activity exhibited by undigested CRP (not shown), the ability to bind $(^3H)d(I\text{-}C)_n$ is lost. Shown in Figure 3 is the titration of $(^3H)d(I\text{-}C)_n$ with CRP, SAP-CRP and ∝CRP. The increased affinity of the CRP-cAMP complex for DNA is best observed at low CRP to DNA ratios; high CRP concentrations allow significant cAMP-independent binding. In contrast to the

hyperbolic binding curve of cAMP-dependent DNA binding by CRP, the CRP cAMP-independent binding curve is sigmoidal. The binding curves may reflect a difference in the cAMP-dependent vs cAMP-independent binding mechanisms which are presently not understood. SAP-CRP and αCRP bind relatively little $(^3H)d(I\text{-}C)_n$ in the absence or presence of cAMP suggesting that the polypeptide regions lost from these CRP cores may be involved in both specific and non-specific components of CRP DNA binding function.

C) Sulfhydryl Content.

The sulfhydryl content of SAP-CRP was determined by DTNB titration (Ellman, 1959) based on a molecular weight of ~36,000 daltons for a dimeric SAP-CRP core. SAP-CRP does not contain the available sulfhydryl groups present in the DNA binding region of CRP (Table I.). Denaturation with SDS has given an ambiguous DTNB titration of 2 to 3 moles SH per mole SAP-CRP core. The presence of 3 SH groups in SAP-CRP would suggest that following proteolysis, one of the available SH groups of CRP is retained but becomes unavailable as a result of some alteration or refolding of the β region. These results could be interpreted to indicate that the available SH groups of CRP reside between two SAP cleavage sites which are in close proximity such that cleavage at either of the two sites would not alter the observed M.Wt. on SDS-polyacrylamide gels. An asymmetric SAP cleavage could then explain the retention of the SH group on some SAP-CRP subunits.

D) Bio Rex 70 Chromatography.

The charge properties of CRP, αCRP and SAP-CRP were

examined by chromatography on the cation exchange resin, Bio Rex 70, a procedure which has previously shown that αCRP is less positively charged than CRP (Eilen et al., 1978). CRP, αCRP and SAP-CRP, retained by Bio Rex 70 at pH 7, resolve at different points along a 0 to 0.5 M KCl gradient (Fig. 4). SAP-CRP elutes at 0.19 M KCl followed by αCRP at 0.3 M KCl and CRP at 0.36 M KCl. These values are in agreement with the NaCl concentrations used to preparatively separate SAP-CRP (∼0.22 M NaCl) from CRP (∼0.34 M NaCl) as described under "Materials and Methods". The significantly lower salt concentration required to elute SAP-CRP from the Bio Rex 70 column indicates that SAP-CRP has a lower net positive charge than either CRP or αCRP at pH 7. These data suggest that SAP-CRP contains a higher ratio of acidic/basic amino acids than CRP or αCRP.

E) Proteolytic Studies.

The effect of cAMP on the proteolytic digestion of CRP and the SAP-CRP core is observed in Figure 5. Unlike CRP, the SAP-CRP core is sensitive to proteolysis even in the absence of cAMP. Digestion of the SAP-CRP core in the absence and presence of cAMP yields polypeptides of ∼13,000 daltons which correspond to the core regions generated from native CRP in the presence of cAMP. The ability of cAMP to stabilize the 13,000 dalton fragments against further digestion is especially apparent when the SAP-CRP is subjected to subtilisin digestion. cAMP has been shown to increase the resistance of the α core to proteolysis and denaturation

(Eilen and Krakow, 1977a). The increased stability of the proteolytic fragments derived from SAP-CRP in the presence of cAMP supports the assumption that the SAP-CRP core contains the cAMP-binding core region of CRP.

F) Limited Peptide Mapping by Proteolysis in SDS.

A simple method of peptide mapping by limited proteolysis in SDS (Cleveland et al., 1977) was employed to compare CRP, αCRP and SAP-CRP. SDS-chymotrypsin digestion of CRP and the SAP-CRP core yields two fragments: CRP: 12,000 $\pm$ 500 and 9000 $\pm$ 500 daltons; SAP-CRP: 9500 $\pm$ 500 and 9000 $\pm$ 500 daltons. Chymotrypsin digestion of αCRP in the presence of SDS yields only the 9000 dalton fragment (Fig. 6). Since less than 20% of the protein is lost by proteolysis (not shown), the 12,000 dalton fragment derived from CRP and the 9,500 dalton fragment derived from SAP-CRP may represent at least part of the DNA binding domain.

The DNA binding region of CRP contains two available SH groups (Eilen and Krakow, 1977a; Eilen et al., 1978). Labeling these groups with a radioactive SH reagent prior to chymotrypsin digestion of CRP would provide a means of identifying which of the fragments corresponds to the DNA binding region. Treatment of CRP with (^{14}C)NEM under native and denaturing conditions resulted in the binding of 2 and 4 moles of (^{14}C)NEM respectively. After thorough dialysis, the samples were digested with chymotrypsin in the presence of SDS. Following gel electrophoresis of the digests, fragment bands were excised and the protein eluted and

counted as described in Table II. When CRP is labeled with (^{14}C)NEM under native conditions, 80% of the label is found in the 12,000 dalton fragment, the remainder is present in the 9000 dalton fragment, indicating that the 12,000 dalton fragment contains the available SH groups. When all four SH residues are titrated with (^{14}C)NEM under denaturing conditions, the label is almost equally distributed between the 12,000 and 9,000 dalton fragments of CRP. As may be predicted by comparison of the electrophoretic patterns following digestion of CRP and αCRP in SDS (Fig. 6), the 9,000 dalton fragment appears to be derived from the cAMP binding domain of CRP containing the cysteinyl residues which are only accessible to SH reagents under denaturing conditions (Eilen and Krakow, 1977a).

Shown in Figure 7 are the fragment patterns produced by subtilisin digestion in SDS. At 15 minutes, digests of CRP contain a $\sim$12,000 dalton fragment while SAP-CRP digests show $\sim$12,000 and 11,000 dalton fragments. These higher molecular weight fragments, which are almost totally digested by 60 minutes, may represent part of the DNA binding region which is absent from αCRP. After 60 minutes digestion, three coincident size classes of fragments are observed in digests of CRP, αCRP and SAP-CRP: 9750 $\pm$ 250, 8250 $\pm$ 250 and 7250 $\pm$ 250 daltons. Several factors render it difficult to make definitive statements regarding the derivation of these fragments: The fragments must represent partially overlapping segments of CRP as evidenced by the sum in excess of their molecular weights and their non-

stoichiometric production. Conversion of larger fragments into stable lower molecular weight species is not observed; continued digestion results in the gradual degradation of all fragments (not shown). It is possible that potential subtilisin cleavage sites are initially unavailable but once fragments are produced they become more susceptible; this may result from alterations of residual conformational structure of the protein in SDS (Tanford, 1958). Protein determinations (not shown) indicate that 80% of CRP, 90% of αCRP and 75% of SAP-CRP remain after 60 minutes digestion. Since αCRP comprises only 60% of CRP (Krakow and Pastan, 1973), additional fragments are expected to be present in digests of CRP and SAP-CRP. If, however, fragments are produced which coincide in size with fragments from the core region, they would not be discerned. Alternatively, small polypeptides may be formed which are undetectable in this gel system. Comparison of CRP, αCRP and SAP-CRP digests indicate that the relative proportions of the fragments produced after 60 minutes are not the same. For example, in αCRP and SAP-CRP digests the 9750 $\pm$ 250 dalton fragment is present in greater quantity than the 8250 $\pm$ 250 dalton fragment while digests of CRP show a higher proportion of the 8250 dalton fragment. This observation, more obvious in densitometry tracings which are not presented here, may reflect a different pattern of subtilisin digestion of CRP compared with αCRP and SAP-CRP or may indicate that the CRP derived 8250 dalton fragments are heterogenous representing segments of the αcore region and the β region

of CRP.

The products of digestion by SAP are shown in Figure 8. SAP digestion of CRP yields a small proportion of two fragments of 13,000 ± 500 and 12,000 ± 500 daltons which are not present in digests of SAP-CRP. A major band of 11,500 ± 500 daltons is produced by SAP digestion of CRP. A band of slightly lower molecular weight, 10,500 daltons, is present in SAP-CRP digests. It is possible that this 10,500 dalton fragment represents a portion of the 11,500 dalton fragment produced from CRP. Two coincident bands of 9500 ± 500 and 8250 ± 250 daltons, observed in electrophoretic patterns of CRP, αCRP and SAP-CRP digests may represent segments of the αCRP core region. An additional faint band representing a fragment of 7500 ± 250 daltons is present in αCRP digests. Protein determinations indicate that 70% of CRP, αCRP and SAP-CRP protein remain after 60 minutes (not shown). Again, the sum of the fragments indicates that they probably represent overlapping regions of CRP.

2. Studies of CRP Crosslinked by Phenylenedimaleimides[1,2]

[1] Structures of bifunctional reagents are presented in Figure A-2.

[2] Throughout this study CRP crosslinked with pPDM was compared with oPDM crosslinked CRP. Unless otherwise indicated, it can be assumed that no significant differences exist between oPDM and pPDM crosslinked CRP.

Treatment of CRP with oPDM in the presence or absence of cAMP, leads to the crosslinking of the two CRP subunits as shown by SDS-polyacrylamide gel electrophoresis. Densitometric tracings of CRP samples treated with a 10 fold excess of oPDM for 90 minutes at 37°C in the absence (B) or presence (C) of 0.1 mM cAMP, show the disappearance of the 22,500 dalton CRP subunit band (A) and the appearance of a 45,000 dalton band corresponding to two crosslinked subunits (Fig. 9). Samples of CRP crosslinked in the absence of cAMP show an additional faint band of slightly lower mobility, observed as a split band (B), the nature of which is presently not understood. Neither oPDM nor pPDM were able to produce intersubunit crosslinks in αCRP or the SAP-CRP core (not shown).

A) Inter-protomer vs Intra-protomer Crosslinks.

To determine whether the crosslinked subunits were derived from 2 CRP protomers as opposed to an intra-protomer crosslink, samples determined to be 95-100% crosslinked were applied to a Sephadex G-100 column (Fig. 10). CRP crosslinked in the presence or absence of 0.1 mM cAMP eluted in the same position as unmodified CRP, indicating that the crosslink occurs within a CRP protomer and not between two protomers. Supporting the conclusion that oPDM modification yield intraprotomer crosslinks are comparable results obtained by sucrose density centrifugation and the observation that crosslinking is independent of CRP concentration (not shown).

B) Factors Affecting the Rate of Crosslinking.

Increased PDM concentrations effect higher yields of crosslinked CRP (Fig. 11). While high concentrations of PDM might be expected to favor monofunctional substitution of CRP reactive groups, concentrations of PDM as high as 60 fold the CRP concentration did not decrease the extent of crosslinking. These results could occur if the crosslinking reaction occurs rapidly subsequent to PDM incorporation or if one mole of PDM sterically prevents the incorporation of a second molecule. The ability of maleimides to react with NH_2 groups as well as SH groups (Brewer and Riehm, 1967) would allow more than one crosslink to form. The results in Fig. 11 also suggest that pPDM crosslinks CRP at a lower rate than oPDM, especially in the absence of cAMP.

The rate of crosslinking by oPDM and pPDM in the presence and absence of cAMP was compared by calculating the half-time (t 1/2) for the initial rate of the crosslinking reaction (Table III.); a representative semi-logrithmic plot of the percent of uncrosslinked CRP vs time is shown in Figure 12. The data presented in Table III indicate that cAMP enhances the rate of subunit crosslinking at 10 fold molar ratios of both oPDM and pPDM to CRP. In the absence of cAMP, the crosslinking rate by a 1 to 1 molar ratio of PDM to CRP is significantly lower for pPDM (t 1/2, 50 minutes) than for oPDM (t 1/2, 15 minutes); this disparity in the crosslinking rate diminishes when CRP is treated with a 10 fold excess of the PDMs or when cAMP is present during

the crosslinking reaction.

The rate of crosslinking varies with cAMP concentration (Figure 13). At 1 to 1 molar ratios of oPDM to CRP, the rate of the crosslinking reaction is considerably enhanced at 5×10^{-6} M cAMP.

Analogues of cAMP such as cGMP or 5'AMP which do not produce conformational transitions required for DNA binding (Krakow, 1975) do not stimulate the rate of crosslinking by oPDM (Table IV). Cyclic TuMP, an active analogue of cAMP with repect to stimulation of gal operon transcription (Nissley et al., 1971) and production of conformational change in CRP (Krakow, 1975; Wu and Wu, 1974), also increases the rate of crosslinking. In the experiment shown in Table IV , the presence of 10^{-4} M cTuMP increased the amount of crosslinked CRP to 80% from 35%, observed in the absence of added ligands. A similar increase in the proportion of crosslinked CRP occurs in the presence of 10^{-3} M cAMP. The ability of cTuMP to effect a conformational change in CRP at concentrations approximately 10 fold lower than cAMP has been previously observed (Krakow, 1975).

The rate of crosslinking by oPDM in the presence of cAMP is decreased by poly d(A-T) or poly d(I-C) (Figure 14), while the yield of crosslinked CRP produced after a 5 minute incubation of oPDM with CRP in the absence of cAMP is relatively unaffected by the deoxypolymers. (not shown). The interference of the deoxypolymers with the crosslinking reaction in the presence of cAMP could result if oPDM reacts within the DNA binding region of CRP or if the

deoxypolymers alter the conformation of the CRP-cAMP complex to one which is less favorable for crosslinking.

C) Activity Studies.

The functional properties of oPDM crosslinked CRP were studied (Table V). Control samples, subjected to the same treatment as modified CRP, show characteristic cAMP binding properties (Anderson et al., 1971). The enhanced ability of CRP to bind DNA in the presence of cAMP is evidenced by a 9 fold increase in the retention of (^{3}H) d(I-C)$_n$ on nitrocellulose filters by the CRP-cAMP complex. CRP modified in the absence of cAMP appears to have a lower affinity for cAMP, reflected by a 3 to 4 fold higher dissociation constant, and may bind more than one cAMP molecule. CRP modified in the presence of cAMP shows a similar dissociation constant and number of cAMP binding sites as unmodified CRP. Inhibition of cAMP-dependent DNA binding is not as complete as that observed for samples treated in the absence of cAMP. Although differences in the nature of CRP modified with oPDM in the presence of cAMP compared with CRP modified in the absence of cAMP are suggested, the data primarily indicate that oPDM modified CRP loses DNA binding activity while cAMP binding remains relatively unaffected.

Conditions employed in these experiments would permit the reaction of maleimides with NH_2 groups as well as SH groups (Brewer and Riehm, 1967). To ascertain their involvement with oPDM crosslinks, the available SH groups of CRP were titrated with the specific SH reagent, DTNB (Eilen and

Krakow, 1977b), prior to oPDM modification. Pretreatment of CRP with DTNB completely blocks the formation of oPDM crosslinks suggesting that cysteinyl residues may be involved in the crosslinks (Table VI). While DTNB modification can be reversed by SH reagents, oPDM modification is irreversible. Prior to assay of CRP function, all samples described in Tables V and VI were treated with mercaptoethanol to remove excess reagents, displace the TNB groups and to reduce any DTNB mediated disulfide bonds (Eilen and Krakow, 1977b). Comparison of oPDM modified CRP (Table V) with oPDM treated CRP, pretreated with DTNB (Table IV), shows that DTNB protects against the loss of $(^3H)d(I\text{-}C)_n$ binding activity exhibited by oPDM modified samples. The presence of TNB or a disulfide bond in the DNA binding region of CRP (Eilen and Krakow, 1977b) appears to block oPDM modification, preventing the loss of DNA binding activity and the formation of crosslinks. Monofuctional substitution by oPDM in other regions may account for the inability of DTNB to prevent the loss of cAMP binding activity resulting from oPDM treatment in the absence of cAMP. The lower affinity of these samples for cAMP does not affect their ablility to bind $(^3H)d(I\text{-}C)_n$ presumably since excess cAMP is used in the assay mixtures.

D) Proteolytic Studies.

While oPDM treated CRP demonstrates significant cAMP binding activity, the question remains as to whether modified CRP shows a functional response to cAMP. Proteolysis has proved a useful probe for examining cAMP induced conformational change in CRP (Krakow and Pastan , 1973; Krakow,

1975; Eilen and Krakow, 1977a) and was therefore employed to similarly investigate oPDM crosslinked CRP.

Figures 15 and 16 show the chymotryptic digestion pattern of CRP crosslinked in the absence or presence of cAMP. Native CRP digested in the presence of cAMP is shown in the last lane of Figure 15 while CRP digested in the absence of cAMP is shown in the last lane of Figure 16. A 13,000 dalton CRP core is produced only in the presence of cAMP; in the absence of cAMP CRP is resistant to proteolysis. Likewise, CRP crosslinked in the absence of cAMP remains resistant to proteolysis after incubation with chymotrypsin for 60 minutes. The presence of 0.1 mM cAMP during proteolysis effects the production of an approximately 20,000 dalton fragment and a 13,000 dalton fragment which corresponds to the CRP core. The 20,000 dalton fragment, absent in unmodified CRP digests, must be a consequence of crosslinking.

CRP which has been crosslinked in the presence of cAMP (Fig. 16) remains susceptible to chymotrypsin digestion in the absence of cAMP; only the 13,000 dalton fragment is generated. The presence of 0.1 mM cAMP during proteolysis increases the rate of digestion but does not induce the production of additional fragments.

Similar results have been observed following subtilisin or trypsin digestion of crosslinked CRP. The densitometric fragment patterns in Figure 17 show the relative resistance to proteolysis in the absence of cAMP, of CRP crosslinked

in the absence of cAMP (A) and the susceptibility of CRP crosslinked in the presence of cAMP(B). The presence of cAMP during proteolysis of CRP crosslinked in the absence of cAMP (A) results in the production of ~15,000 and 12,000 dalton tryptic fragments and 12,000 and ~11,000 dalton subtilisin generated fragments. In contrast to chymotrypsin digestion of crosslinked CRP, fragments of similar molecular weight are generated by trypsin digestion of CRP crosslinked in the absence (A) and presence (B) of cAMP . CRP crosslinked in the presence of cAMP is nearly completely digested by subtilisin; the presence of cAMP during proteolysis appears to stabilize the 12,000 dalton subtilisin fragment.

The proteolysis experiments indicate that cAMP can induce conformational change in crosslinked CRP. Chymotrypsin digestion of CRP crosslinked in the absence of cAMP results in the production of 20,000 and 13,000 dalton fragments only when concentrations of cAMP of 10^{-5} M or higher are present during proteolysis (Fig. 18). Relatively high concentrations of cAMP increase the rate of digestion of CRP crosslinked in the presence of cAMP, although substantial digestion occurs in its absence (Fig. 18). cGMP cannot replace cAMP in altering the sensitivity of crosslinked CRP to proteolysis (not shown).

E) Limited Peptide Mapping by Proteolysis in SDS.

The proteolysis experiments imply that the presence of cAMP during oPDM modification affects the nature of the crosslink. The crosslinked samples were therefore studied

by limited proteolysis in SDS (Cleveland et al., 1977). In Figures 19 and 20, distinct chymotryptic fragment patterns of crosslinked CRP are observed depending upon whether cAMP had been present during oPDM modification. Protein determinations indicate that less than 25% of the protein is lost following SDS-chymotrypsin digestion (not shown). At early time points, 0.5 to 30 minutes, digests of CRP crosslinked in the absence of cAMP (Fig. 19) show prominent high molecular weight bands of ∼30,000 and 23,000 daltons; less visible are bands of 34-36,000 daltons and a band of ∼27,000 daltons. Two smaller polypeptide fragments of 12,000 and 9000 daltons are produced at the early time intervals. In comparison, digestion of CRP crosslinked in the presence of cAMP (Fig. 20) from 0.5 to 30 minutes generates 3 major fragments of 34,000, 27,000 and 9,000 daltons; the 23,000 dalton and 11,000 dalton fragments are only slightly visible. As proteolysis progresses from 60 to 300 minutes, CRP crosslinked in the absence of cAMP breaks down into 3 stable fragments of ∼23,000, 12,000 and 9,000 daltons. Beginning at 60 minutes, digests of CRP crosslinked in the presence of cAMP show the gradual decrease of the 34,000 dalton band while two new bands of 23,000 and 12,000 daltons appear and increase in amount with time; after 300 minutes, 4 fragments remain : 27,000, 23,000, 12,000 and 9,000. In another experiment (not shown), an additional 24 ug of chymotrypsin, added after 180 minutes, did not lead to further digestion of fragments observed in Figures 19 and

20. While fragments of similar size have been generated in digests of CRP crosslinked in the absence and presence of cAMP, the rate of appearance and stability of these fragments differ.

As previously described, chymotrypsin digestion of unmodified CRP in SDS generates two stable fragments of 9000 ± 500 daltons from the core region and 12,000 ± 500 daltons from the β region of CRP (Fig.6; Figs. 19 and 20, last lane). These fragments coincide in size with the lower molecular weight fragments appearing in digests of crosslinked CRP. In digests of CRP crosslinked in the absence (Fig. 19) and presence (Fig. 20) of cAMP, the 9000 dalton polypeptide appears to be the only fragment produced at the same rate in both samples. If, as the evidence suggests, oPDM modification occurs within the β region of CRP, cleavage of sites within the CRP core region should not be affected. The apparently unimpeded production of the 9000 dalton fragment from both crosslinked samples suggests that it is derived from the core region of CRP. It is difficult to suggest a possible origin of the 12,000 dalton fragment which is produced at different rates from CRP crosslinked in the presence or absence of cAMP. Since the 12,000 dalton polypeptide formed from native CRP contains the two available SH groups (Table II), one or both of which appear to be involved in the oPDM crosslink, it is reasonable to assume that the 23,000 and 27,000 dalton fragments represent two crosslinked peptides from the β region. This being the case, the 12,000

dalton fragment must be derived from either another segment of CRP or must be a product of further digestion of the larger fragments, perhaps composed of two crosslinked polypeptides.

Limited proteolysis of crosslinked CRP in SDS using Staph.aureus protease, like chymotrypsin digestion, yields fragment patterns which differ depending upon whether cAMP was present during oPDM modification (Fig. 21). SAP, which cleaves peptide bonds on the carboxyl side of aspartyl or glutamyl residues, shows a greater specificity for glutamyl residues in buffers containing ammonium ions (Houmard and Drapeau, 1972). Digestion in Tris-HCl buffer produces fragments of $\sim$21-25,000 daltons from CRP crosslinked in the absence of cAMP and 15-18,000 daltons from CRP crosslinked in the presence of cAMP; fragments of $\sim$13,000, 9500 and 8000 daltons appear in both crosslinked digests. The greater specificity of SAP in ammonium bicarbonate buffer limits the number of cleavage sites generating several higher molecular weight fragments. Digests of CRP crosslinked in the absence of cAMP show fragments of $\sim$30,000, 25,000 and 13,000 daltons while digests of CRP crosslinked in the presence of cAMP show fragments of $\sim$42,000, 35,000, 28,000 and 16,000 daltons.

Subtilisin and papain also generate different fragment patterns from CRP crosslinked in the absence and presence of cAMP (not shown).

G) Crosslinking by Other Reagents.

Studies using MBS, a heterobifunctional reagent which reacts with SH and NH_2 groups (Kitagawa and Aikawa, 1976), show that NH_2 and SH groups of CRP are capable of being crosslinked in the presence and absence of cAMP. Like PDM crosslinked CRP, MBS crosslinked CRP retains cAMP binding activity but no longer binds (^3H) deoxypolymers (not shown). Densitometric tracings of SDS-polyacrylamide gel electrophoretic patterns of MBS-crosslinked CRP digested by chymotrypsin in the presence and absence of cAMP are shown in Figure 22. Examination of undigested samples (A) appears to indicate that MBS crosslinks are favored in the absence of cAMP since a significant amount of uncrosslinked CRP remains in samples crosslinked in the presence of cAMP. This result, which has been repeatedly observed, is an interesting contrast to PDM crosslinking which is enhanced by the presence of cAMP. Like oPDM crosslinked CRP, CRP crosslinked with MBS in the absence of cAMP is relatively resistant to proteolysis while CRP crosslinked in the presence of cAMP is very susceptible to proteolytic attack (B). In the presence of 0.1 mM cAMP, chymotrypsin digestion of CRP crosslinked with MBS in the absence and presence of cAMP produces two major fragments of $\sim$20,000 and 13,000 daltons; these fragments are similar in size to those produced from CRP crosslinked with oPDM in the absence of cAMP (D), although more extensive digestion of MBS-crosslinked CRP is observed. SDS-chymotrypsin digestion of MBS-crosslinked CRP produces fragment

patterns which are similar to those produced from CRP cross-linked with oPDM in the absence of cAMP (Figure 23).

Modification of CRP with another reagent capable of bridging SH and NH_2 groups, N-(3-pyrene)maleimide (Wu et al., 1976),did not result in the formation of crosslinks although a loss of DNA binding activity was observed (not shown).The inability of pyrene maleimide to crosslink the CRP subunits presumably reflects the spacial orientation of the SH and NH_2 groups. The pyrene maleimide crosslink involves the aminolysis of the succinimide derivative which results from the SH addition to the maleimide, requiring close proximity of the NH_2 and SH groups (Wu et al., 1976). MBS crosslinks involve two functional groups, a succinimide and a maleimide residue, which are spacially more distant (Kitagawa and Aikawa, 1976).

Attempts to crosslink lysyl residues of CRP with dimethylsuberimidate (Davies and Stark, 1970) have been unsuccessful (not shown). Incubation with dimethylsuberimidate did not result in the loss of CRP functional activity.

3. Modification of CRP with TNBS.

CRP was modified with trinitrobenzenesulfonic acid (TNBS) to investigate the role of lysine residues in the binding of CRP to DNA. TNBS reacts with unprotonated

amino groups, at pH 8-9, to yield trinitrophenylated (TNP) residues (Okuyama and Satake, 1960). Initial experiments which attempted to measure the rate of TNBS modification of CRP vs CRP-cAMP complexes were abandoned when spectrophotometric analysis of TNBS titrations revealed that TNBS modified CRP-cAMP complexes formed precipitates. Instead, TNBS modified CRP was prepared as described under "Materials and Methods" and soluble protein was analyzed.

A) Spectrophotometry.

Shown in Figure 24 is an absorption spectrum of TNBS modified CRP. The absorption maxima at 347-348 nm and a shoulder at about 420 nm are indicative of TNP-amino group derivatives (Hollenberg et al., 1971).

B) DNA Binding Activity.

CRP modified with TNBS according to the procedure described in "Materials and Methods" contains approximately 6-7 TNP-lysine residues, as determined spectrophotometrically, and retains 65-70% of the (^{3}H)cAMP binding activity of unmodified CRP (not shown). Titration of (^{3}H)d(A-T)$_n$, in the presence and absence of cAMP, with unmodified and TNP-CRP indicates that both cAMP-dependent and cAMP-independent binding of CRP to (^{3}H)d(A-T)$_n$ is inhibited by modification of 6 or 7 out of 26 total CRP lysine residues (Fig. 25A). In contrast, the TNP-CRP retains $\sim$70% of the cAMP-dependent (^{3}H)d(I-C)$_n$ binding activity exhibited by unmodified CRP while only 25% of the cAMP-independent binding activity remains

(Fig. 25B). The differences in the ability of TNP-CRP to bind the two deoxypolymers may reflect the manner in which CRP interacts with the promoter site.

DISCUSSION

The SAP-CRP core shares several properties with other proteolytic cores derived from CRP in the presence of cAMP (Krakow and Pastan, 1973; Eilen et al., 1978): SAP-CRP has lost the capacity to bind $(^3H)d(I\text{-}C)_n$ while retaining (^3H)-cAMP binding activity (Fig. 3); cAMP is able to stabilize the 13,000 dalton SAP-CRP limit core against further proteolytic digestion (Fig. 5); SAP-CRP contains the buried SH groups present in α CRP (Table I). Since the α CRP retains the dimeric structure of CRP, these results strongly suggest that the SAP-CRP core consists of two 18,000 $\pm$ 500 dalton fragments which represent the NH_2- proximal region of CRP. The polypeptide region lost following SAP digestion must represent all or part of the COOH-terminal DNA binding domain of CRP. Analysis of NH_2 and COOH- terminal amino acids as well as sedimentation velocity studies will definitively characterize the SAP-CRP core.

A schematic diagram of CRP, SAP-CRP and α CRP is presented in Figure 26. The two α CRP fragments, accounting for the double bands observed in SDS-polyacrylamide gels of this α CRP preparation (Figs. 6-8), presumably reflect non-uniform subtilisin cleavage of CRP subunits. The CRP cores have been aligned at the NH_2-terminus since α CRP has been shown to have the same NH_2-terminal amino acid sequence ($\pm$ an NH_2-terminal dipeptide) as CRP (Schlesinger, 1978). SDS-chymotrypsin cleavage sites have been placed to give a

9000 dalton fragment from CRP, SAP-CRP and αCRP and a 12,000 dalton fragment from CRP (Fig. 6); this arrangement also accounts for the 9500 dalton fragment derived from SAP-CRP. According to this diagram, polypeptides of $\sim$4000 daltons should be present in α CRP digests. While fragments of this size are not observed, it is possible that such fragments are unstable, allowing further proteolysis. The placement of the SH groups is based on DTNB titrations of CRP, α CRP and SAP-CRP and from experiments in which CRP was modified with (^{14}C)NEM under native and denaturing conditions, prior to SDS-chymotrypsin digestion (Table II). (^{14}C)NEM labeling experiments indicate that both the 12000 and 9000 dalton fragments contain SH groups; the SH group present in the 9000 dalton fragment is only marginally labeled using non-denaturing conditions. As both of the native SAP-CRP subunits do not contain SH groups which are reactive with DTNB, the SH group within the 12,000 dalton fragment must be placed after the SAP-CRP COOH-terminus. The SH groups within the 9000 dalton fragment are arbitrarily placed. Comparison of the charge properties of CRP, SAP-CRP and αCRP reveals that while removal of a $\sim$10,000 dalton polypeptide from the COOH-terminus of CRP, to yield αCRP, produces a significant decrease in net positive charge, it is not nearly as substantial as that observed for SAP-CRP, which lacks only a 4000 dalton fragment. These results suggest that the 4000 dalton fragment, lost from SAP-CRP, contains a relatively high concentration of positively charged amino acids. In

CRP, the positively charged residues may be partially balanced by a region which contains a relatively high proportion of acidic amino acids; this region is present in SAP-CRP but absent from ∝CRP. The fact that further incubation of SAP-CRP with SAP results in the production of a 13,000 dalton limit core (not shown) supports the hypothesis that glutamyl and aspartyl residues are located in the COOH-terminal region of SAP-CRP. The ionic properties of SAP-CRP can thus be explained by the loss of a positively charged region combined with the retention of a negatively charged region of CRP.

Limited proteolysis in SDS (Cleveland et al., 1977) provides a means of easily obtaining and visualizing fragments of CRP. SDS-chymotrypsin digestion has proven to be useful for analysis of CRP and CRP cores as it appears that few of the 12 possible chymotrypsin cleavage sites (Anderson et al., 1971) are available in this system and one of the cleavage sites apparently separates the functional domains of CRP. Digestion of CRP with subtilisin or SAP in the presence of SDS produces more complicated sets of fragments which are difficult to interpret (Figs. 7 and 8). The subtilisin and SAP generated fragments may prove useful, however, in conjunction with chemical modification experiments in which CRP and CRP cores are differentially labeled, in the absence and presence of cAMP or using native and denaturing conditions, prior to proteolysis. Fragments of interest can then be isolated electrophoretically for further analysis.

The production of the SAP-CRP core and its charge properties indicate that glutamyl & aspartyl amino acid residues are present in the DNA binding domain of CRP. Lancelot and Hélène (1977) have shown that carboxylic acids interact strongly and specifically with guanine. It is noteworthy in this respect that the CRP binding site is rich in G·C base pairs (Dickson et al., 1975) and that point mutations which decrease CRP binding (Majors, 1975a) involve G·C to A·T transitions (Dickson et al., 1977). Acidic amino acids may be involved in the specific interaction of CRP with its DNA binding site. Loss of cAMP-dependent and independent DNA binding activity might also be attributed to the decrease in the positive charge of SAP-CRP which suggests that electrostatic interactions may also mediate the binding of CRP to DNA.

Study of the SAP-CRP core has placed the available SH group of CRP close to the COOH-terminus; assuming an average amino acid molecular weight of 150, it is estimated that the SH is between 10 to 30 amino acids from the COOH end of CRP.

Phenylenedimaleimides were employed to probe the conformational shift of the available cysteine residues of CRP. Both oPDM and pPDM proved capable of crosslinking CRP in the presence and absence of cAMP even though the functional groups of pPDM span at least twice the 5 Å distance which separates the functional groups of oPDM (Chang and Flaks, 1972). Like the DTNB mediated disulfide bonds (Eilen and Krakow, 1977b),

PDM crosslinks occur between two subunits of a CRP protomer (Fig. 10). The conformational state induced by cAMP and its active analogue, cTuMP. appears more favorable for the crosslinking reaction by both oPDM and pPDM (Tables III and IV). The shift of the SH groups to a less polar environment when cAMP binds to CRP (Wu,F.Y.-H., et al., 1974) may account for an increased reactivity of the aromatic PDM with CRP. The proximity of the residues to be bridged by oPDM would also influence the crosslinking rate. The DTNB studies of Eilen and Krakow (1977b) indicate that in the presence of cAMP the two SH groups come within 2 Å of each other allowing the formation of a disulfide bond between the two CRP subunits. Assuming that pPDM also reacts with the two SH groups, or residues within the same microenvironment, the ability of pPDM to form a 12-14 Å crosslink suggests a certain degree of flexibility between the two polypeptides of the β region. Following similar reasoning,the significantly lower rate of crosslinking in the absence of cAMP by pPDM compared with oPDM implies that the spacial distance between the two sulfhydryl groups may be approaching a limit of ∼14 Å in the absence of cAMP. Valid delineation of polypeptide chain movements can only be made when the specific amino acids crosslinked by the ortho and para PDMs have been determined.

The PDM crosslinks reside within or near the β region of CRP which is involved in DNA binding function. PDM modification of CRP results in the loss of DNA binding activity while cAMP binding function is retained (Table V).

DTNB, known to react with the SH groups located in the β-region (Eilen and Krakow, 1977a&b), blocks the formation of oPDM crosslinks and prevents the irreversible loss of DNA binding function (Table VI). The αCRP and SAP-CRP cores, which lack all or part of the DNA binding domain, are not crosslinked by ortho or para PDM and polydeoxynucleotides have been shown to decrease the rate of oPDM crosslinking (Fig. 14).

The maintenance of cAMP binding activity indicates that PDM modification did not alter the overall conformation of CRP. Furthermore, proteolytic digestion experiments demonstrated that cAMP was able to elicit conformational transitions in crosslinked CRP.

PDMs have proved to be useful probes for investigating conformational transitions within the β region of CRP. In the absence of cAMP, the DNA binding domain of CRP appears to be closely associated with the core region, explaining its resistance to proteolytic attack (Krakow and Pastan, 1973). cAMP may act to alter the conformation of the DNA binding region permitting interaction with DNA and access to proteolytic enzymes. In the presence of cAMP, oPDM appears to crosslink CRP in a manner which prevents the reassociation of the DNA binding region with the core domain after cAMP has been removed by dialysis. CRP crosslinked in this manner remains susceptible to enzymatic attack regardless of whether cAMP is present during proteolysis (Fig. 16). CRP crosslinked in the absence of cAMP retains its resistance to

proteolysis ; upon addition of functionally significant levels of cAMP during proteolysis, the β region polypeptides become available and digestion occurs producing the 13,000 dalton CRP core and a 20,000 dalton fragment which probably represents a crosslinked peptide (Fig. 15).

On the basis of their sensitivity to protease digestion, the two crosslinked preparations can be described as having structural distinctions which reflect the conformational state of CRP by virtue of their dependence upon the absence or presence of cAMP during PDM modification. Other observations also suggest differences in the nature of CRP crosslinked in the absence of cAMP compared with CRP crosslinked in the presence of cAMP; CRP modified in the absence of cAMP shows a slight decrease in its affinity for cAMP (Table V) and displays a split band in electrophoretic patterns (Fig. 9). The distinct peptide patterns produced by digestion of the two crosslinked samples in the presence of SDS (Figs. 19 and 20) provide further evidence that the PDM crosslinks have stabilized different conformational structures of CRP.

PDM modification could generate particular conformational structures by crosslinking different combinations of amino acids in the absence and presence of cAMP. Experiments where CRP was pretreated with DTNB prior to oPDM modification (Table VI), indicate a cysteinyl residue as at least one of the functional groups involved in the crosslink. This assumes that the effect of DTNB is not due to steric hindrance or altered charge properties of the β region by

incorporated TNB. While neutral pH favors the specific reaction of maleimide reagents with SH groups, reaction with amino groups accur at more alkaline pH. PDM modification of CRP was performed at pH 8, where the cAMP-dependent DNA binding activity of CRP is observed (Krakow and Pastan, 1973). Since a CRP protomer contains 26 lysyl and 2 available cysteinyl residues as well as 2 NH_2-terminal amino groups (Anderson et al., 1971), a number of potential crosslinks could be formed. Studies of the SAP-CRP core indicate that the available cysteinyl residues are located within a positively charged region of CRP. The ability of the heterobifunctional reagent, MBS, to form crosslinks between CRP subunits implies that amino groups are potentially within range for the formation of SH-NH_2 crosslinks by PDMs. The similar electrophoretic digestion patterns of CRP crosslinked with MBS in the absence and presence of cAMP (Figs. 22 and 23) and CRP crosslinked with oPDM in the absence of cAMP infer that the cAMP-independent oPDM crosslink may involve a SH-NH_2 crosslink.

Consideration should be given to the possibility that both cAMP-dependent and independent PDM crosslinks involve the two available cysteinyl residues of CRP and whether such crosslinks can generate structural diversity. Freedburg and Hardman (1971) have observed that functional heterogeneity exists in preparations of E.coli tryptophan synthetase in which two specific cysteinyl thiol residues were crosslinked by bis(maleimidomethyl)ether. They proposed that four

different conformational forms of the crosslink may be generated by the introduction of new asymmetric centers resulting from addition of the protein thiols across the two maleimide bonds of the bifunctional reagent; the order of addition of the two cysteinyl thiols to the bis(maleimide) may direct the stereochemical course of the reaction. A similar mechanism could be proposed for the formation of PDM crosslinks by assuming that the initial reaction of one of the two SH groups of CRP is differentially affected by the absence or presence of cAMP. Whatever the structural distinctions rendered by the PDM crosslinks, proteolysis experiments indicate that they are maintained in the presence of SDS.

The outcome of protease digestion in a denaturing system would be expected to differ if PDM modification in the absence or presence of cAMP occurs at different amino acid residues, particularly if the modified residues are located near proteolytic cleavage sites. Structural differences which remain in the presence of SDS may also affect the susceptibility of the crosslinked preparations to protease digestion. Conformational studies reveal that a large degree of ordered structure can be found in proteins denatured by SDS (Tanford, 1968; Reynolds and Tanford, 1970). Also, disulfide bonds have been shown to decrease the binding of SDS to protein (Pitt-Rivers and Impionbato, 1968). Thus, if the conformational structures generated by the oPDM crosslinks can in turn affect the denatured state produced by SDS, differential susceptibility to proteolysis may result.

Although fragments which are likely to contain cross-linked peptides have been identified, the electrophoretic patterns produced after proteolysis in SDS yield limited information regarding the position of the cAMP-dependent and cAMP-independent crosslinks. More rigorous techniques of peptide mapping are required to clarify the nature of the crosslinks. The extent of modification should also be investigated as monofunctional substitutions and intra-polypeptide crosslinks may occur. The present work indicates that efforts to locate the positions of the oPDM, pPDM and MBS crosslinks should provide useful information regarding the conformational transitions of CRP.

Positively charged amino acid residues may contribute to the binding of CRP to DNA through electrostatic interactions with the phosphate groups on the DNA backbone. The coordinate loss of positive charge and DNA binding function from CRP digested with proteolytic enzymes (Figs. 3 and 4) suggests that lysine, arginine or histidine residues are located within the DNA binding domain. CRP was modified with TNBS to acertain the importance of lysine residues for DNA binding function; TNBS modification converts the positively charged ϵ-amino groups of lysine to neutral TNP-amino residues. Lysine residues have been shown to play a role in the formation of DNA complexes with other DNA-binding proteins; acetylation of the lysine residues in the filamentous bacteriophage gene 5 protein decreases its ability to bind single-stranded DNA, (Anderson et al., 1975),

when 40% of the lysine residues of the lac repressor are modified with acetylimidazole, operator binding is abolished (Alexander et al., 1977) and in vivo acetylation of histone lysine residues has been correlated with changes of chromatin structure and function (Sung and Dixon, 1970; Ruiz-Carrillo et al., 1975). Under conditions where cAMP binding activity is relatively unaffected, treatment of CRP with TNBS such that approximately seven lysine residues are trinitrophenylated, inhibits the formation of cAMP-dependent and independent $(^3H)d(A\text{-}T)_n$-CRP complexes. In contrast, cAMP-dependent binding of TNP-CRP to $(^3H)d(I\text{-}C)_n$ remains relatively unaltered while significant reduction of cAMP-independent binding occurs (Fig. 25). The differential ability of TNP-CRP to bind the two deoxypolymers may reflect the manner in which CRP interacts with the promoter site. In this respect, it is interesting to note that native CRP-cAMP complexes show greater affinity for $d(I\text{-}C)_n$ than $d(A\text{-}T)_n$ (Krakow and Pastan, 1973) and the CRP interaction site within the lac operon contains regions of alternating G·C base pairs (Dickson et al., 1975). Since $d(I\text{-}C)_n$ appears as a normal G·C copolymer in the major groove, it is possible that $d(I\text{-}C)_n$ mimics the lac operon binding site. Evidence suggesting that CRP does, in fact, contact the major helical groove comes from observations which indicate that CRP has a higher affinity for DNA containing halogen substituents in the 5-position of the pyrimidine ring, exposed in the major groove (Lin and Riggs, 1976). The more 'specific'

interactions of CRP with $d(I\text{-}C)_n$ compared with $d(A\text{-}T)_n$ might be less affected by TNBS modification. Lac repressor protein modified with N-bromosuccinimide also displays differential DNA binding; mild reaction conditions completely detroys operator DNA binding while binding to non-specific DNA is unaltered (O'Gorman and Matthews, 1977). Though these observations suggest that distinct sites for specific and non-specific DNA binding activities may exist, such distinctions must represent local variations within a single DNA binding domain; the same portions of repressor subunits have been indicated to bind both operator and non-specific DNA (Linn and Riggs, 1975b; Wang, A.C. et.al., 1977) and evidience suggesting that both $d(A\text{-}T)_n$ and $d(I\text{-}C)_n$ bind to the same DNA binding site of CRP includes the ability of both deoxypolymers to protect against proteolysis of the DNA binding domain (Krakow and Pastan, 1973) and the equivalent inhibition of both $d(A\text{-}T)_n$ and $d(I\text{-}C)_n$ binding by SH modification (Eilen and Krakow, 1977b). Comparison of chemically modified specific and non-specific DNA-protein complexes may clarify whether different protein sites are involved in high and low affinity DNA binding activity.

A major difficulty encounterd during the TNBS studies involved the tendency of TNP-CRP to precipitate as solvent interactions presumably decreased when the positive charge of the lysine residues was neutralized by trinitrophenylation. Mild reaction conditions followed by removal of excess TNBS by Bio-Gel chromatography allowed the prep-

aration of soluble TNP-CRP. By further decreasing the reaction time or the TNBS concentration it may be possible to determine the minimum degree of trinitrophenylation which produces a loss of cAMP-dependent DNA binding activity. Very mild reaction conditions may also allow differential TNBS modification of CRP, CRP-cAMP and CRP-cAMP-DNA complexes. Tritium labeled TNP-CRP can also be prepared and digested in the presence of SDS to determine the distribution of modified lysine residues between the α core and β region fragments.

CONCLUDING REMARKS

The properties of SAP-CRP are summarized in Table A-II and a summary of the effects of chemical modification on CRP is presented in Table A-III. The main conclusions of this work can be summarized as follows:

1. Study of the SAP-CRP core has provided new insights regarding native CRP structure:

a) The $\sim$4000 dalton polypeptide region lost following SAP digestion is required for DNA binding function and may represent all or part of the COOH-terminal DNA binding domain of CRP.

b) The presence of glutamyl and aspartyl residues within the DNA binding domain is indicated by the susceptibility of this region to SAP digestion; it has been sug-

gested that these amino acids may play a role in the binding of CRP to the promoter site.

c) The available SH groups of CRP are predicted to reside within 10 to 30 amino acids from the COOH-terminus of CRP.

d) Comparison of the charge properties of CRP, α CRP and SAP-CRP suggest that basic and acidic amino acids may be unevenly distributed within the β region of native CRP.

2. Limited proteolysis of CRP with chymotrypsin in the presence of SDS produces a 9000 dalton fragment derived from the α core region of CRP and a 12,000 dalton fragment derived from the β region. Electrophoretic patterns indicate that fragments containing portions of the α core region and β region are also produced after digestion of CRP with subtilisin and SAP in the presence of SDS.

3. Phenylenedimaleimides have proved to be useful probes for investigating conformational transitions within the DNA binding domain. It is possible that the PDM crosslinks have stabilized different conformational states of CRP.

4. Trinitrophenylation of 6 to 7 lysine residues altered the DNA binding properties of CRP. Binding of CRP to $(^3H)d\ (A\text{-}T)_n$ is totally inhibited while cAMP-dependent $(^3H)d(I\text{-}C)_n$ binding is relatively unaffected. It is suggested that the differential ability of TNP-CRP

to bind the two deoxypolymers may reflect the manner in which CRP interacts with the promoter site.

During the course of this project, several proteolytic fragments of CRP have been generated. These fragments should prove useful for further investigations of CRP structure and function. Experimental approaches using polypeptide fragments to study protein structure include complementation assays which test the ability of the fragments to reconstitute protein function (Zabin and Villarejo, 1975) and immunological techniques that compare the antigenic properties of the fragments and native protein (Sachs et al., 1972; Celeda et al., 1974). Hybrid CRP molecules, formed between native CRP and CRP core subunits, could be used to determine whether two complete β region polypeptide chains are required for DNA binding activity. A model study of this type has been performed using normal and tryptic core subunits of the lac repressor (Geisler and Weber, 1976). The question of whether the DNA binding domain of CRP exists as an independently stabilized structure could be approached if the 12,000 dalton β region polypeptide is purified from CRP digested with chymotrypsin in the presence of SDS. The use of fragments derived by proteolysis of CRP in SDS for biological assays is encouraged by reports which describe the successful reconstitution of protein function following SDS denaturation (Weber and Kuter, 1971), including

the recovery of lac repressor-operator binding activity (Sadler and Tecklenburg, 1976).

It has been suggested that oPDM crosslinked CRP may be used to elucidate the conformational transitions of CRP. Knowing the positions of the cAMP-dependent and independent PDM crosslinks with respect to the amino acid sequence of CRP should prove helpful for determination of CRP tertiary structure in the absence and presence of cAMP. The amino acid sequencing of CRP is currently in progress (Schlesinger, 1978).

The specific interaction of CRP with its promoter region site is an area which requires resolution. The TNBS studies presented here, though preliminary in nature, suggest that chemical modification of CRP could reveal distinctions between specific and non-specific DNA binding mechanisms of CRP. Specific chemical modification of amino acids such as tyrosine, tryptophan, or arginine might be attempted to ascertain their role in DNA binding function. The possibility exists that by molecular cloning adequate quantities of restriction fragments which carry the CRP binding site can be obtained to better study the specific interactions of CRP and chemically modified CRP with the promoter region.

Finally, while this project has not directly elucidated the mechanism of CRP action it is hoped that avenues of investigation have been further evolved. While study of CRP structure and function is important for understand-

ing the mechanisms of gene regulation in bacteria, knowledge gained through characterization of the cAMP and DNA binding domains of CRP should have general implications regarding the role of cAMP in other systems and the nature of protein-DNA interactions.

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Figure 1. Proteolysis of CRP with SAP in the presence of cAMP and cGMP. Digestion mixtures contained (final volume 50 ul): 10 mM sodium phosphate buffer (pH 8), 1 mM EDTA, 0.1 M NaCl, 4mM DTT, 7.5 ug CRP, 1 mM cAMP or cGMP where indicated and SAP as indicated. All incubations were at 37°C for 60 minutes. Proteolysis was terminated by the addition of 10 ul of PMSF to give a concentration of 0.1 mM. The samples were brought to 0.5 % SDS and 10 % glycerol (final volume 100 ul) and heated for 10 minutes at 60°C. Aliquots (40 ul) were resolved by SDS-polyacrylamide gel electrophoresis (12 % gel).

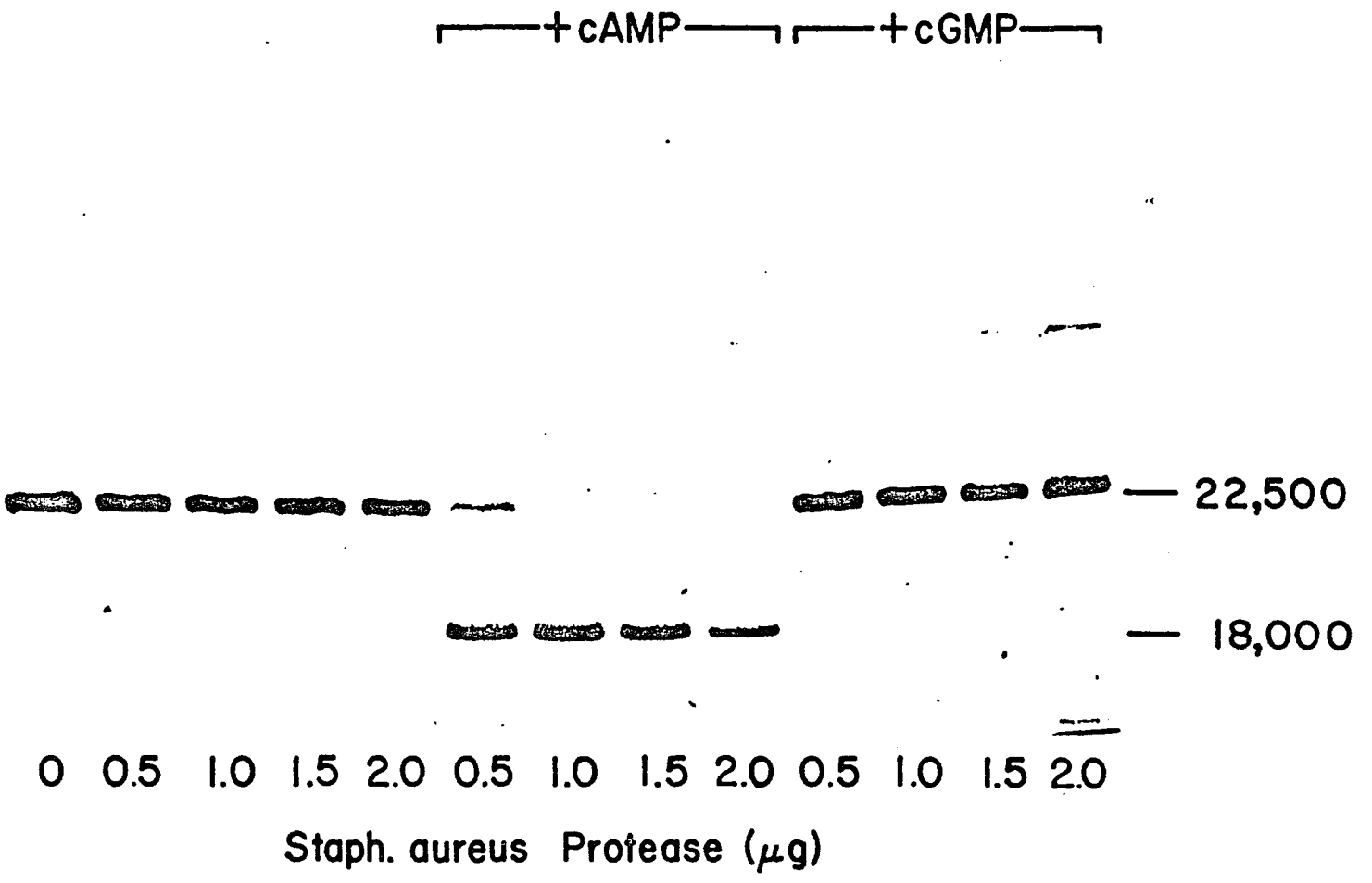
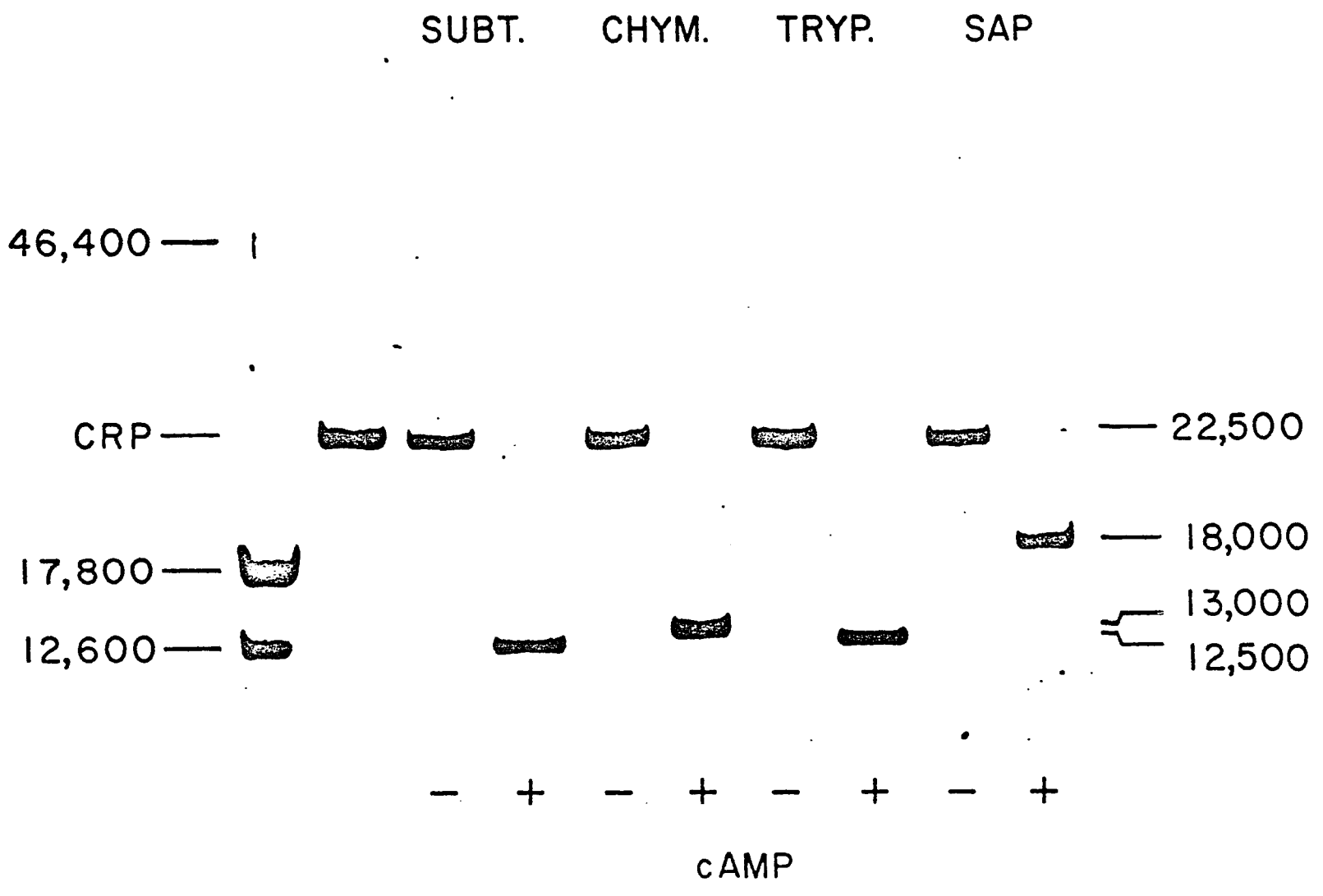

+cAMP
+cGMP
22,500
18,000
0 0.5 1.0 1.5 2.0 0.5 1.0 1.5 2.0 0.5 1.0 1.5 2.0
Staph. aureus Protease (μg)

Figure 2. CRP cores formed following digestion with proteolytic enzymes in the presence of cAMP. Digestion mixtures contained (final volume 50 ul) 21 ug of CRP, 0.1 mM cAMP where indicated and 0.85 ug of subtilisin or 1.7 ug of the other proteases. Digestion with subtilisin or chymotrypsin was performed in 10 mM sodium phosphate (pH 7), 0.1 M NaCl, 1 mM EDTA, and 1mM DTT; when the Staph. aureus V8 protease was used, the above mixture contained 10 mM sodium phosphate, pH 8. Incubation with trypsin was carried out in 50 mM Tris-HCl (pH 8) and 1 mM DTT. All incubations were at 37°C. At the times indicated, proteolysis was terminated by the addition of 5 ul of PMSF to give a concentration of 0.1 mM. The samples were brought to 0.1 % SDS and 20 % sucrose (final volume 200 ul) and heated for 10 minutes at 60°C. Aliquots (40 ul) were resolved by SDS-polyacrylamide gel electrophoresis (12 % slab gel): (a) ovalbumin, mol wt 46,400, myoglobin, mol wt 17,800, cytochrome c, mol wt 12,600; (b) untreated CRP; (c) subtilisin plus CRP (15 minutes); (d) subtilisin plus CRP in the presence of cAMP (15 minutes); (e) chymotrypsin plus CRP (30 minutes); (f) chymotrypsin plus CRP in the presence of cAMP (30 minutues); (g) trypsin plus CRP (30 minutes); (h) trypsin plus CRP in the presence of cAMP (30 minutes); (i) SAP plus CRP (120 minutes); (j) SAP plus CRP in the presence of cAMP (120 minutes).


SUBT.
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46,400
1
CRP
22,500
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17,800
13,000
12,600
12,500
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cAMP

Figure 3. cAMP dependent and independent $(^3H)d(I-C)_n$ binding activity of CRP, SAP-CRP and αCRP. Assay mixtures contained (final volume 0.25 ml): 40 mM BTP (pH 8), 3.5 nmoles $(^3H)d(I-C)_n$ (3490 cpm per nmole), the indicated amount of protein sample minus cAMP (o) or plus 0.4 mM cAMP (•). Mixtures were incubated 5 minutes at 37°C. After addition of 0.75 ml of 50 mM NaCl, the mixtures were filtered onto nitrocellulose membranes which were then dried and counted in Liquifluor-toluene. In the absence of protein, background $(^3H)d(I-C)_n$ binding was 0.11 nmoles; this value was subtracted from all experimental samples.

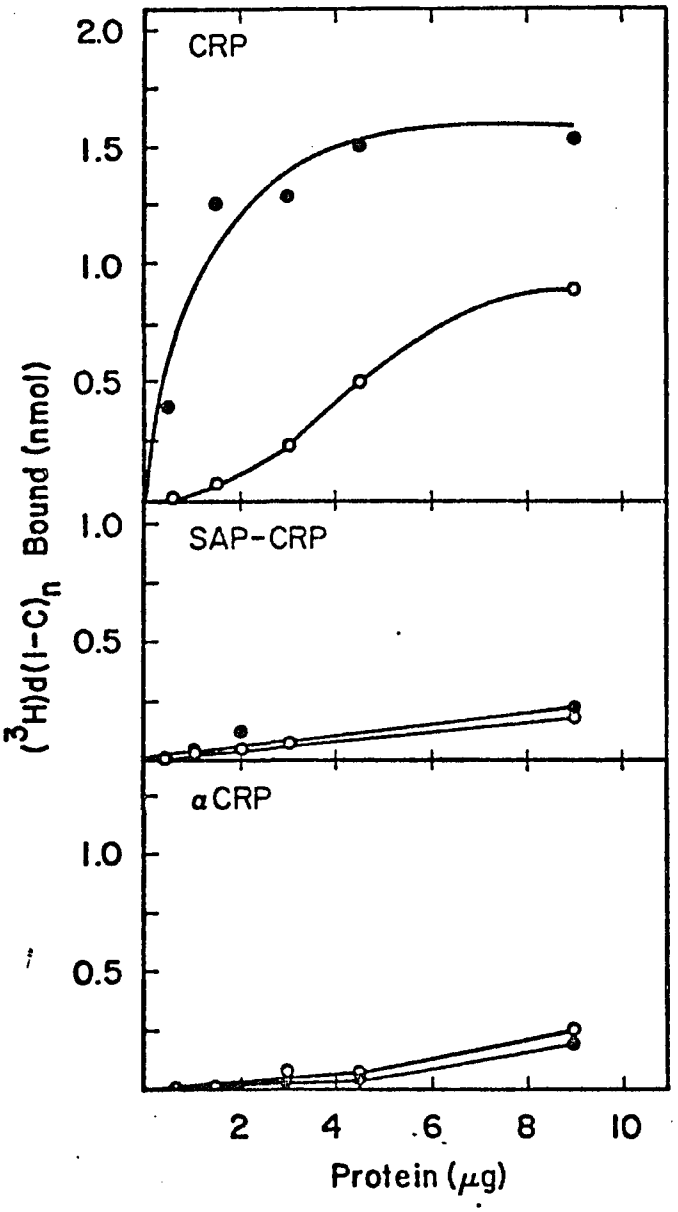

CRP
SAP-CRP
αCRP
2.0
1.5
1.0
0.5
1.0
0.5
1.0
0.5
$(^3H)d(I-C)_n$ Bound (nmol)
2
4
6
8
10
Protein (μg)

Figure 4. Elution of CRP, α CRP and SAP-CRP from Bio Rex 70. Samples containing 0.5 mg of protein in 50 mM potassium phosphate buffer (pH 7) and 0.1 mM EDTA were loaded onto a 1 x 10 cm Bio Rex column and eluted with a 0 to 0.5 M KCl linear salt gradient in the sample buffer (total volume 100 ml). Fractions of 2.0 ml were collected and assayed for protein concentration (o) and (^{3}H)cAMP binding activity (•).

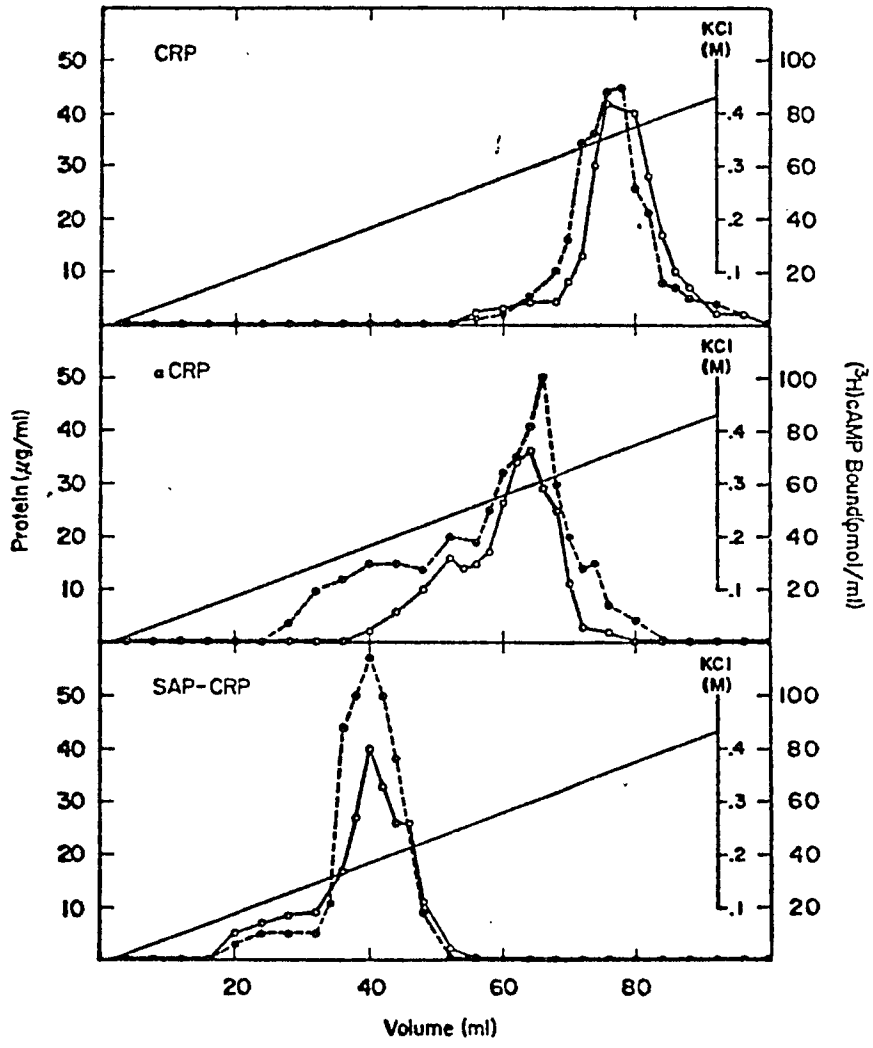

CRP
αCRP
SAP-CRP
KCl (M)
.1
.2
.3
.4
Protein (μg/ml)
(³H)cAMP Bound(pmol/ml)
Volume (ml)
10
20
30
40
50
60
80
100

Figure 5. Effect of cAMP on the proteolysis of CRP and the SAP-CRP core. Digestion mixtures contained (final volume 50 µl): 40 mM BTP (pH 8), 10 mM DTT, 10 µg CRP or SAP-CRP core, 1 mM cAMP where indicated and 0.13 µg of subtilisin or 1 µg αchymotrypsin or trypsin. All incubations were at 37°C. After 10 minutes (subtilisin) or 30 minutes (chymotrypsin or trypsin), proteolysis was terminated by the addition of 10 µl of PMSF to give a final concentration of 0.1 mM. The samples were brought to 0.5% SDS and 10% glycerol (final volume 100 µl) and heated for 10 minutes at 60°C. Aliquots (40 µl) were resolved by SDS-polyacrylamide gel electrophoresis (15% running gel, 4.2% spacer).

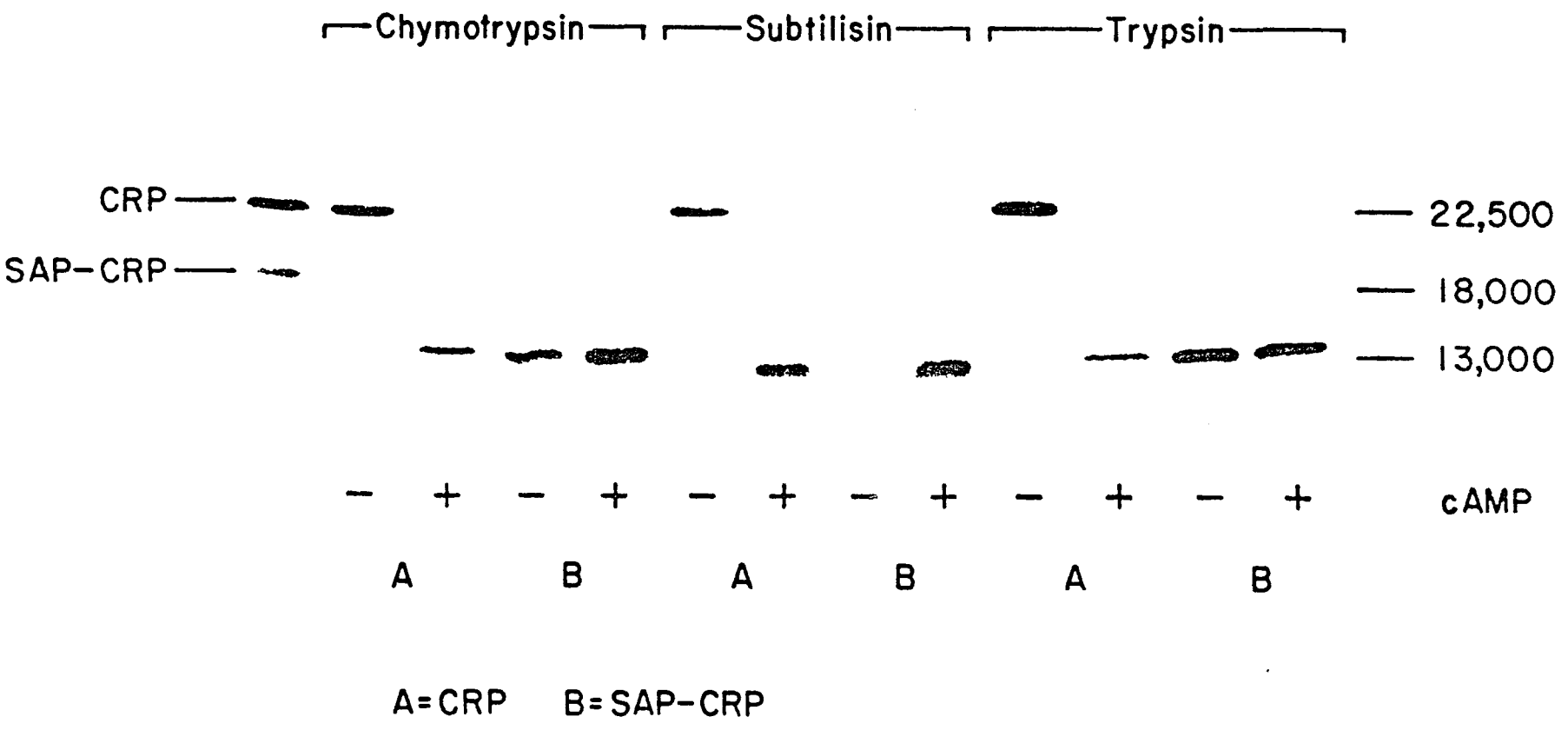
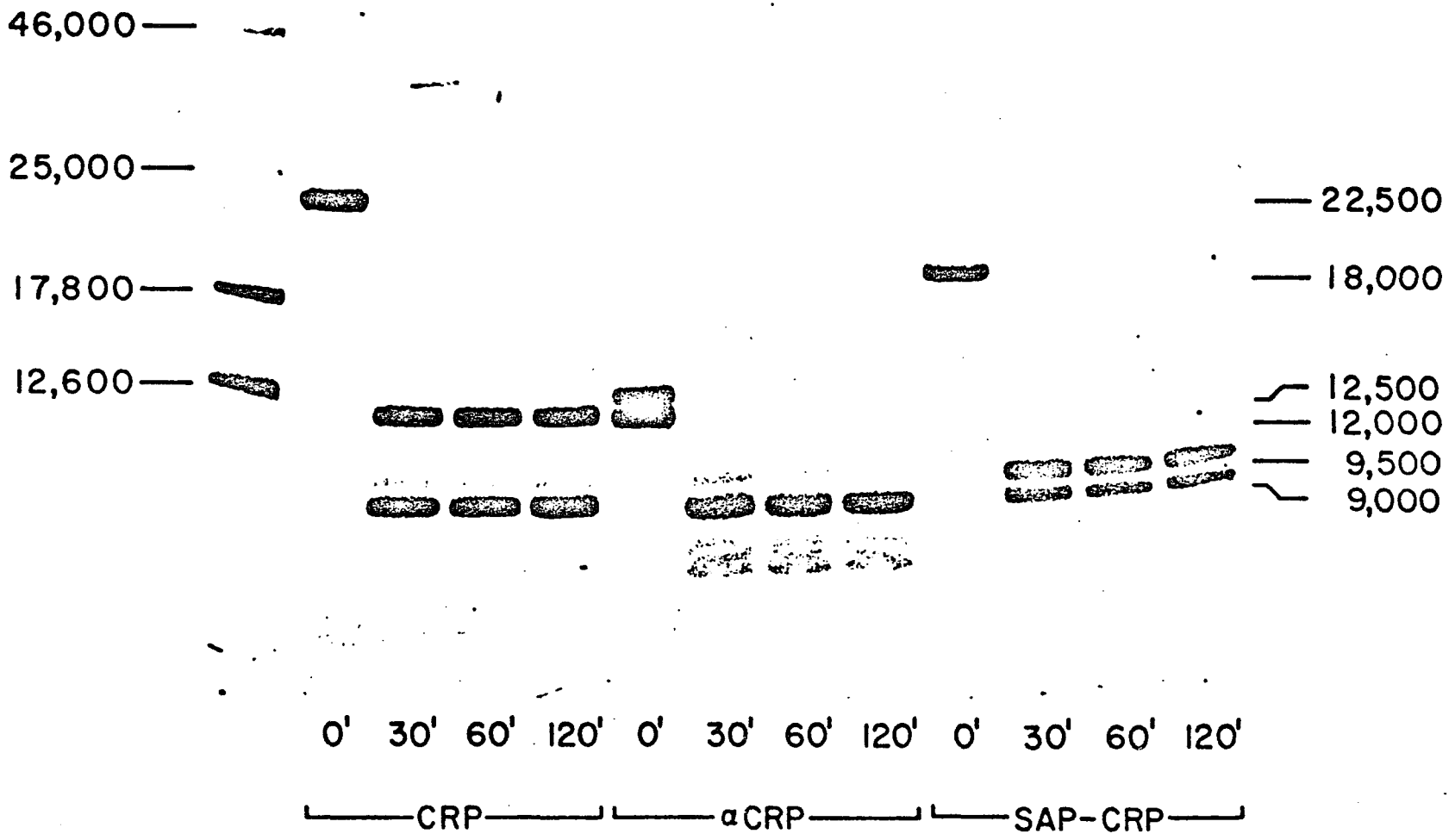
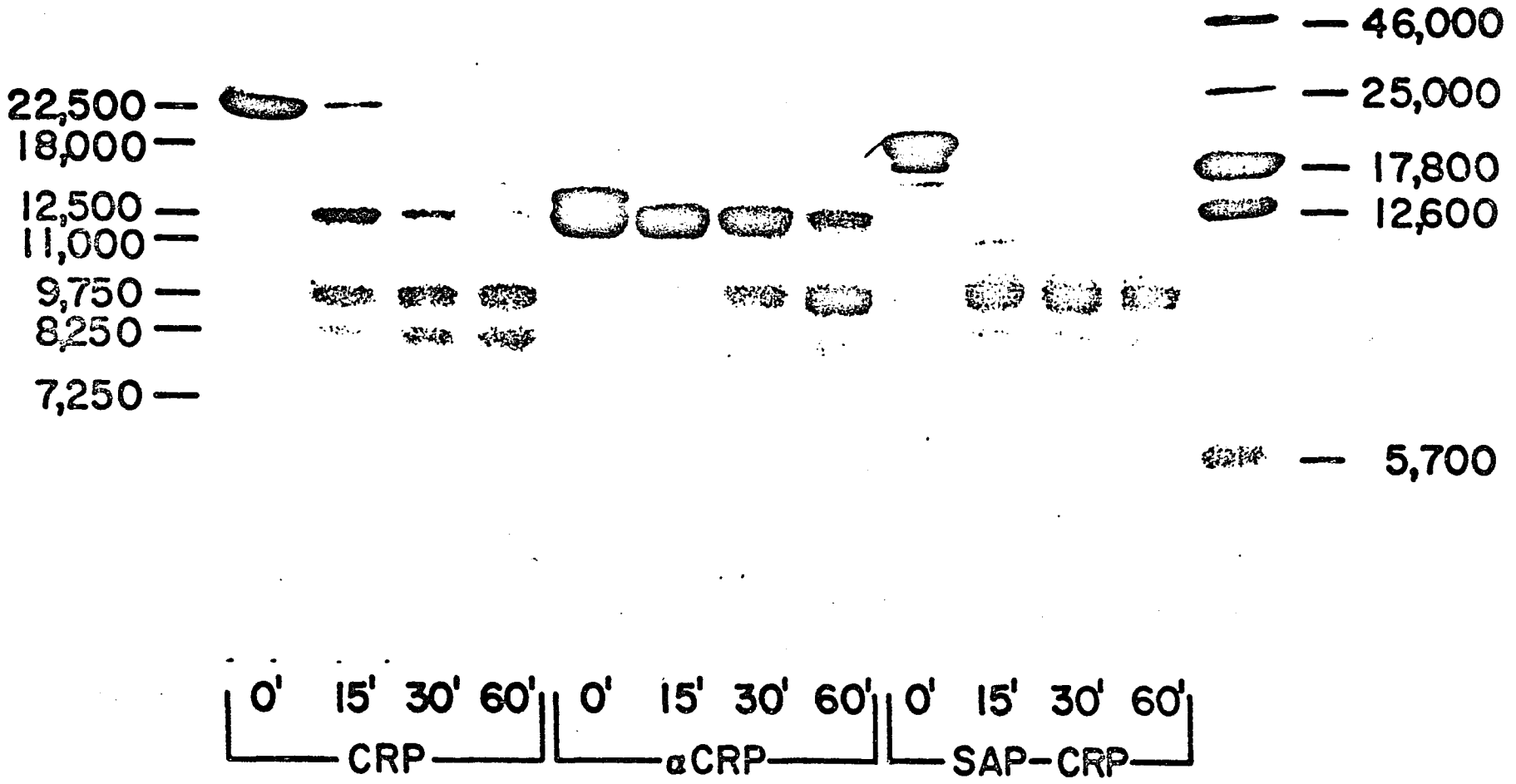

Chymotrypsin
Subtilisin
Trypsin
CRP
SAP-CRP
22,500
18,000
13,000
− + − + − + − + − + − +
cAMP
A B A B A B
A=CRP B=SAP-CRP

Figure 6. Limited proteolysis of CRP, α CRP and SAP-CRP core with α chymotrypsin in SDS. Predigestion mixtures contained (volume 195 ul): 0.125 M Tris-HCl (pH 6.8), 1 mM EDTA, 1 mM DTT, 10 % glycerol, 0.5 % SDS and 30 ug of protein sample. The mixtures were heated in a boiling water bath for 5 minutes. After cooling, 6 ug (in 30 ul) of chymotrypsin was added. Incubations were at 37°C. At the indicated time intervals, proteolysis was stopped by heating in a boiling water bath for 2 minutes. Aliquots (40 ul) were resolved by SDS-polyacrylamide gel electrophoresis (15 % running gel, 4.2 % spacer). Molecular weight standards are shown in the first lane: ovalbumin, mol wt, 46,000; chymotrypsinogen, mol wt 25,000; sperm whale myoglobin, mol wt, 17,800; horse heart cytochrome c, mol wt,12,600. The molecular weights of the proteolytic fragments were determined on 15% SDS-polyacrylamide cylindrical gels as described under "Materials and Methods".


46,000
25,000
17,800
12,600
22,500
18,000
12,500
12,000
9,500
9,000
0' 30' 60' 120' 0' 30' 60' 120' 0' 30' 60' 120'
CRP
αCRP
SAP-CRP

Figures 7. and 8. Limited proteolysis of CRP, α CRP and SAP-CRP core with subtilisin (Fig. 7) and SAP (Fig.8) in SDS. Predigestion mixtures contained (volume 200 ul): 0.125 M Tris-HCl (pH 6.8), 1mM EDTA, 1 mM DTT, 10 % glycerol, 0.5 % SDS and 40 ug of CRP and SAP-CRP and 30 ug of α CRP. The mixtures were heated in a boiling water bath for 5 minutes. After cooling, 0.2 ug (2 ul) of subtilisin (Fig. 7) or 1.5 ug (2 ul) of SAP (Fig. 8) were added. Incubations were at 37°C. At the indicated time intervals, proteolysis was stopped by heating in a boiling water bath for 2 minutes. Aliquots (40 ul) were resolved by SDS-polyacrylamide gel electrophoresis (4.2 % spacer gel; 18 % acrylamide-6 M urea-SDS running gel). The gels were run at 4 ma/slab for 18 hrs. The molecular weights of the proteolytic fragments were determined on 15% SDS-polyacrylamide cylindrical gels as described under "Materials and Methods". Molecular weight standards are shown in the last lane: ovalbumin, 46,000; chymotrypsinogen, 25,000; myoglobin, 17,800; cytochrome c, 12,600; insulin, 5,700.


46,000
25,000
17,800
12,600
5,700
22,500
18,000
12,500
11,000
9,750
8,250
7,250
0' 15' 30' 60'
CRP
0' 15' 30' 60'
αCRP
0' 15' 30' 60'
SAP-CRP

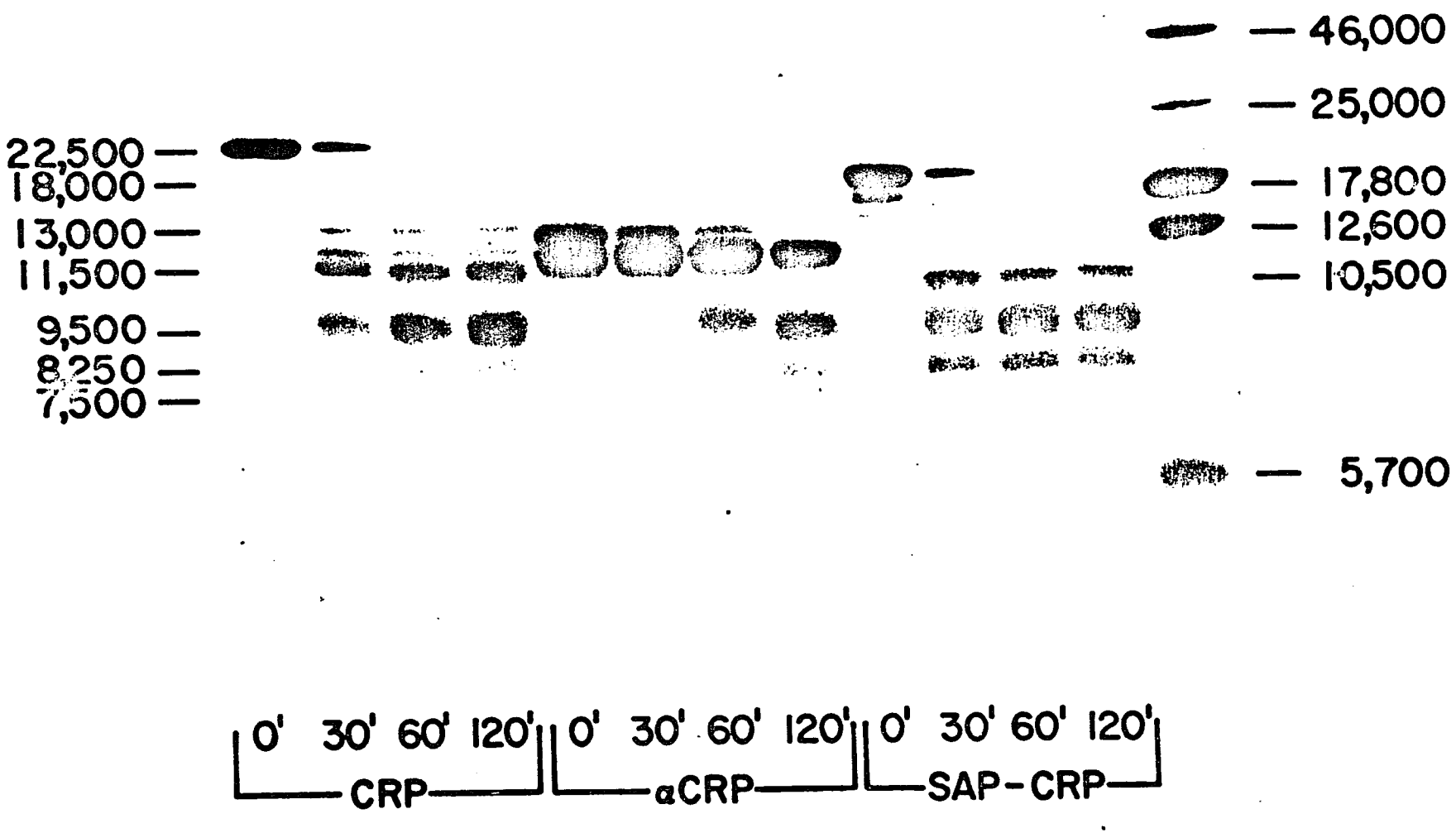

22,500
18,000
13,000
11,500
9,500
8,250
7,500
46,000
25,000
17,800
12,600
10,500
5,700
0' 30' 60' 120'
CRP
0' 30' 60' 120'
αCRP
0' 30' 60' 120'
SAP-CRP

Figure 9. Crosslinking of CRP by oPDM. Samples containing 5 ug of unmodified CRP or CRP modified with oPDM in the prsence or absence of 0.1 mM cAMP, as described under "Materials and Methods", were adjusted to 0.1 % SDS, 15 % sucrose and 1 mM DTT in a final volume of 0.1 ml, incubated for 10 minutes at 60°C and applied in 40 ul aliquots to a 12 % SDS-polyacrylamide slab gel. Electrophoresis and densitometry were performed as described under "Materials and Methods". Unmodified CRP, (A); CRP modified with oPDM in the absence of cAMP, (B); CRP modified with oPDM in the presence of cAMP, (C).

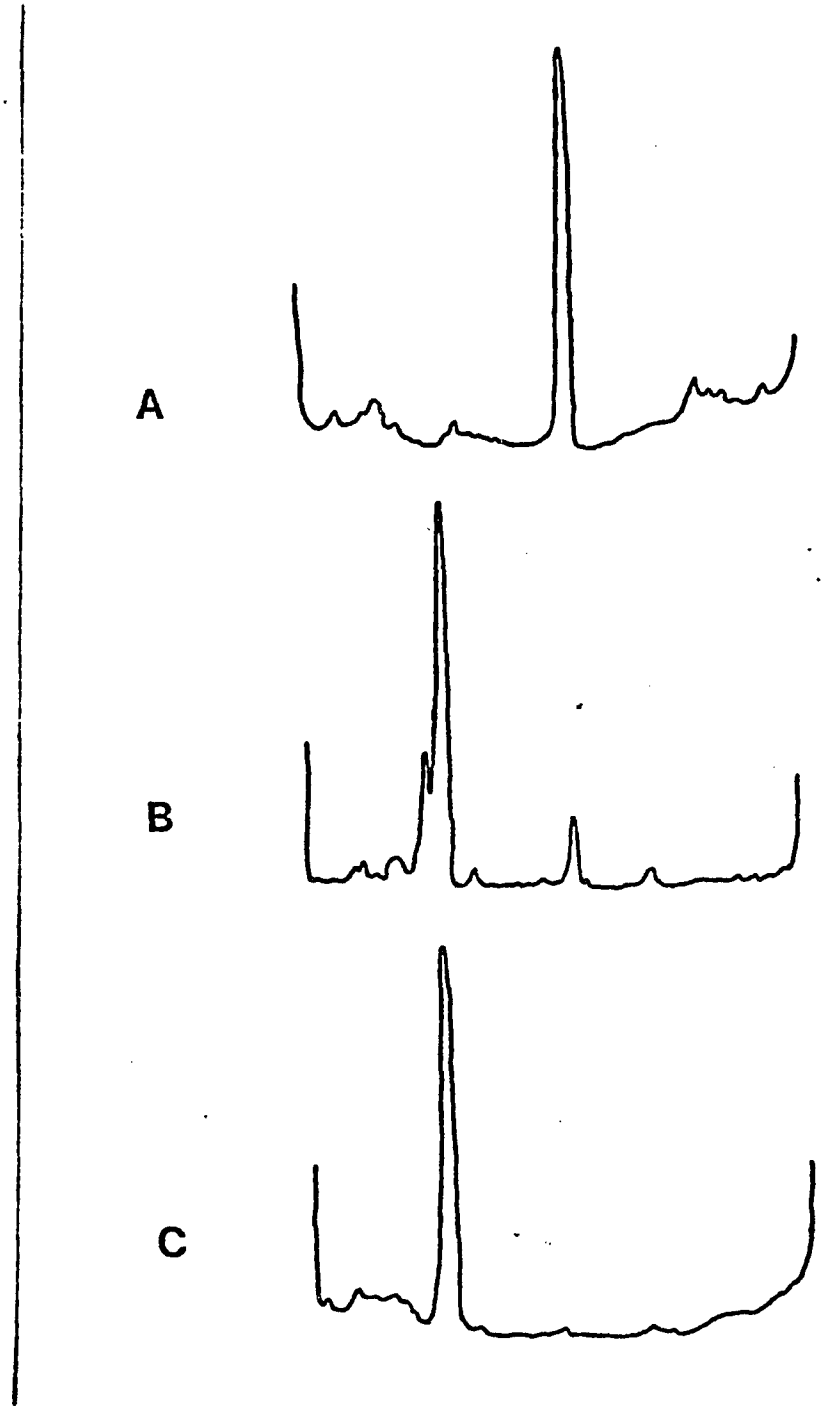

A
B
C

Figure 10. Sephadex G-100 chromatography of unmodified and oPDM crosslinked CRP. Crosslinked samples were prepared as described under "Materials and Methods" with the exception that samples were not dialyzed. Samples containing approximately 0.5 mg of protein in 0.5 ml were applied to a 1 x 55 cm Sephadex G-100 column equilibrated with 10 mM potassium phosphate buffer, pH 7, 1 mM EDTA, 0.1 M NaCl and 1 mM DTT. Fractions of 1 ml were collected and protein determinations performed as described under "Materials and Methods". Unmodified CRP, (▲); CRP crosslinked in the absence of cAMP (o); CRP crosslinked in the presence of 0.1 mM cAMP, (●).

SEPHADEX G-100 CHROMATOGRAPHY OF UNMODIFIED AND o-PDM CROSSLINKED CRP

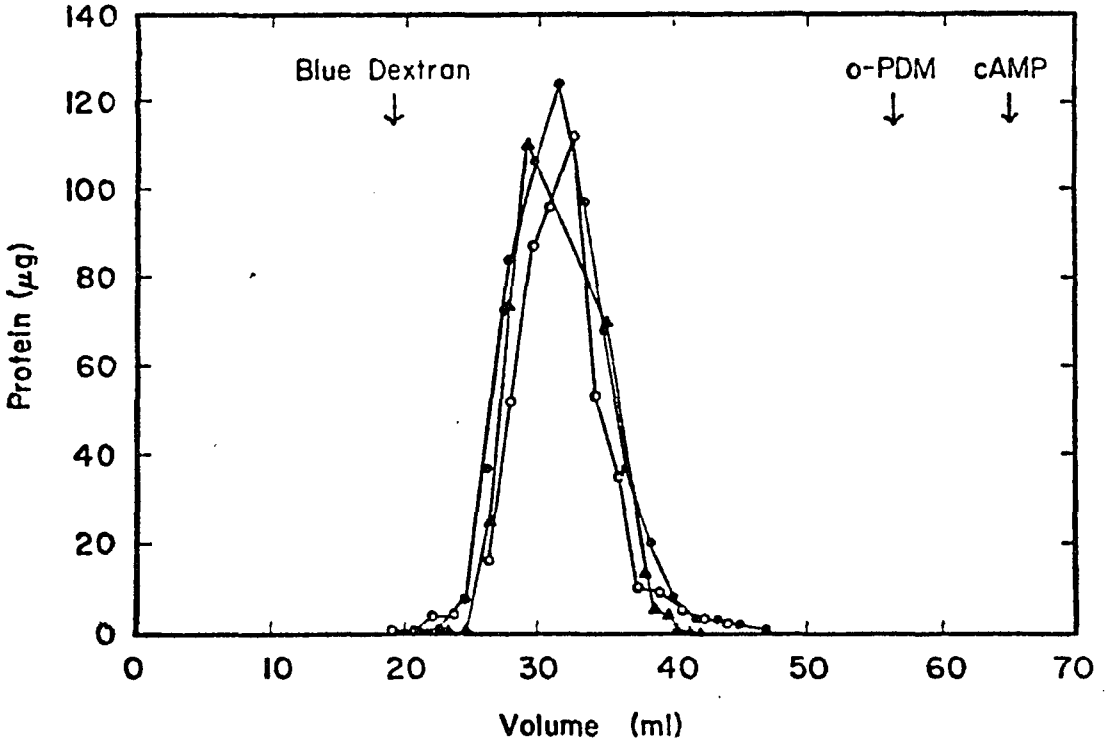


Figure 11. Effect of PDM concentration on the crosslinking rate. The reaction mixtures contained (final volume 50 ul): 5 ug CRP, 40 mM BTP (pH 8), 0.25 mM cAMP where indicated and the indicated concentrations of oPDM or pPDM. After incubation for 10 minutes at 37°C, reactions were terminated by addition of 2 ul of 0.5 M ME. The preparation and resolution of samples by SDS-polyacrylamide gel electrophoresis on 10 % slab gels were performed as described in Figure 9. CRP crosslinked with oPDM: minus cAMP, (o---o); plus cAMP, (●---●). CRP crosslinked with pPDM: minus cAMP,(o——o); plus cAMP, (●——●).

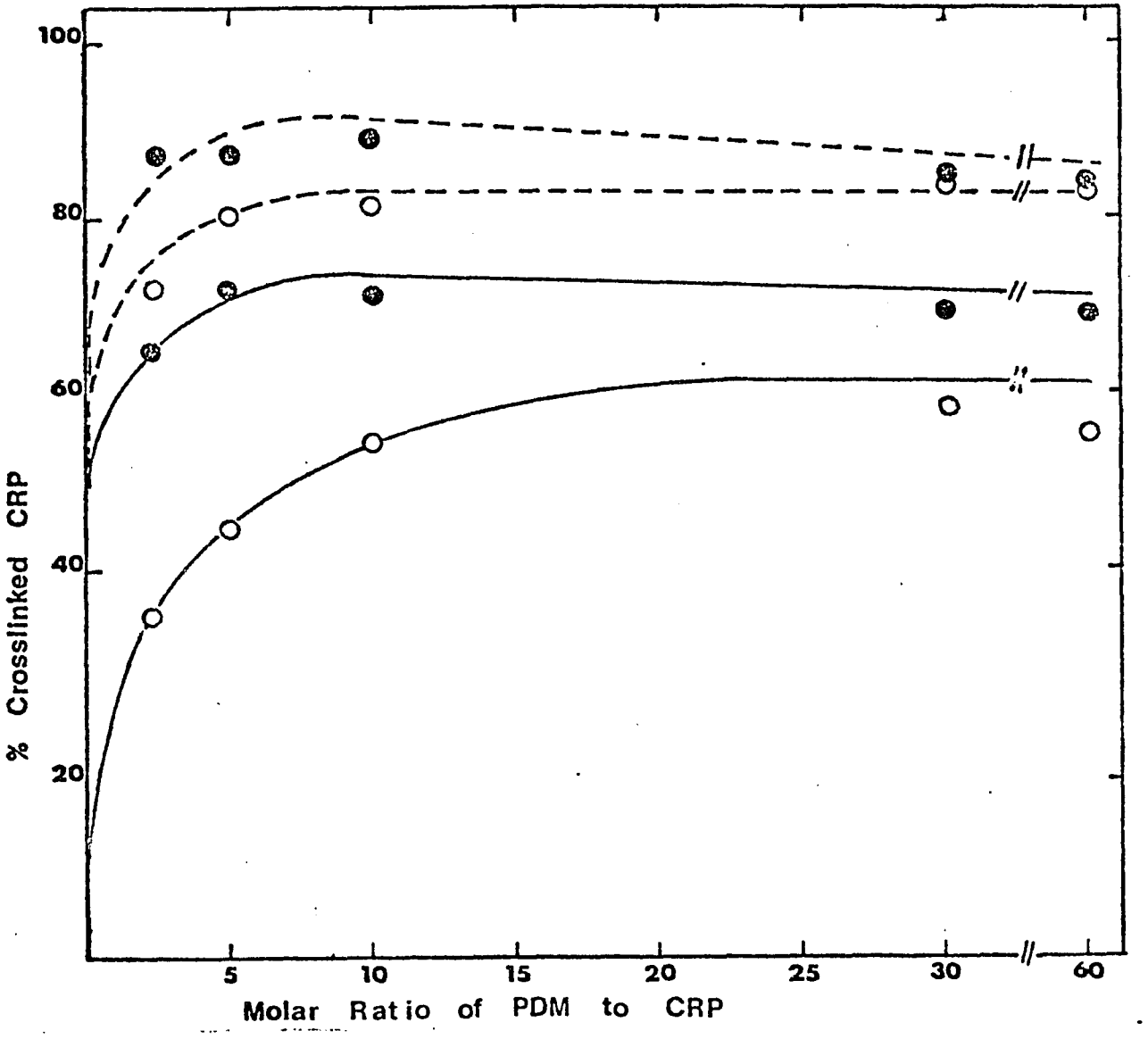

100
80
60
40
20
% Crosslinked CRP
5
10
15
20
25
30
60
Molar Ratio of PDM to CRP

Figure 12. Effect of cAMP on the rate of CRP crosslinking by oPDM. The reaction mixtures contained (final volume 0.5 ml): 50 mM Hepes (pH 8), 110 ug CRP (2.4 nmoles), 25 nmoles oPDM and 0.1 mM cAMP where indicated. The mixtures were incubated at 37°C and at indicated time points 50 ul aliquots were removed and 2 ul of 1 M ME added to terminate the reaction. The preparation and resolution of samples by SDS-polyacrylamide gel electrophoresis on 10 % slab gels were performed as described in Figure 9. CRP crosslinked minus cAMP, (o); CRP crosslinked plus cAMP, (•).

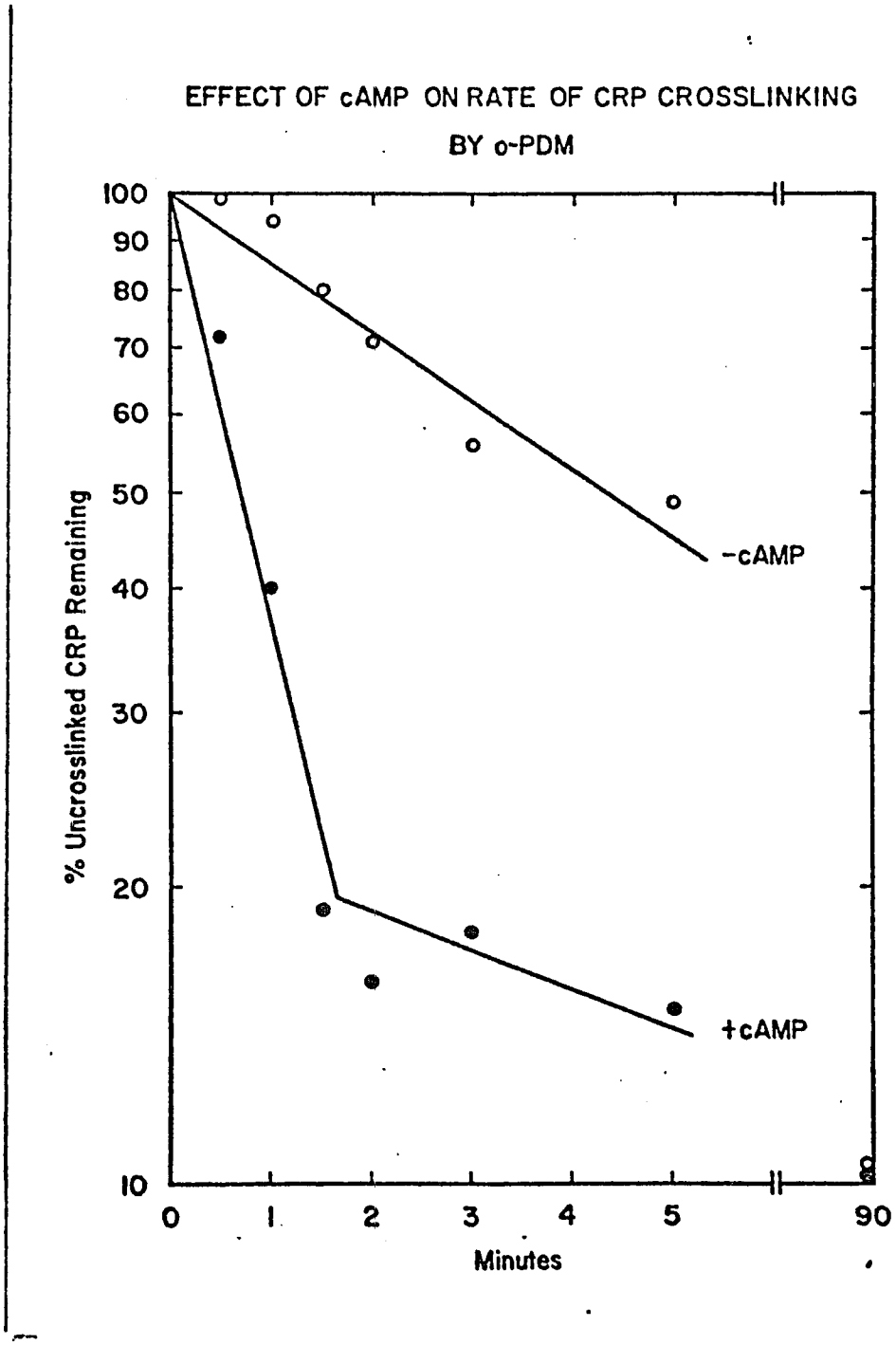

EFFECT OF cAMP ON RATE OF CRP CROSSLINKING
BY o-PDM
% Uncrosslinked CRP Remaining
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
90
Minutes
-cAMP
+cAMP

Figure 13. Crosslinking of CRP with oPDM as a function of cAMP concentration. The reaction mixtures contained (final volume 50 ul) 5 ug CRP (0.11 nmoles), 50 mM Hepes (pH 8) 0.11 nmoles oPDM and the indicated concentrations of cAMP. After incubation for 20 minutes at 37°C, reactions were terminated by the addition of ME to a final concentration of 40 mM. The preparation and resolution of samples by SDS-polyacrylamide gel electrophoresis on 10 % slab gels were performed as described in Figure 9.

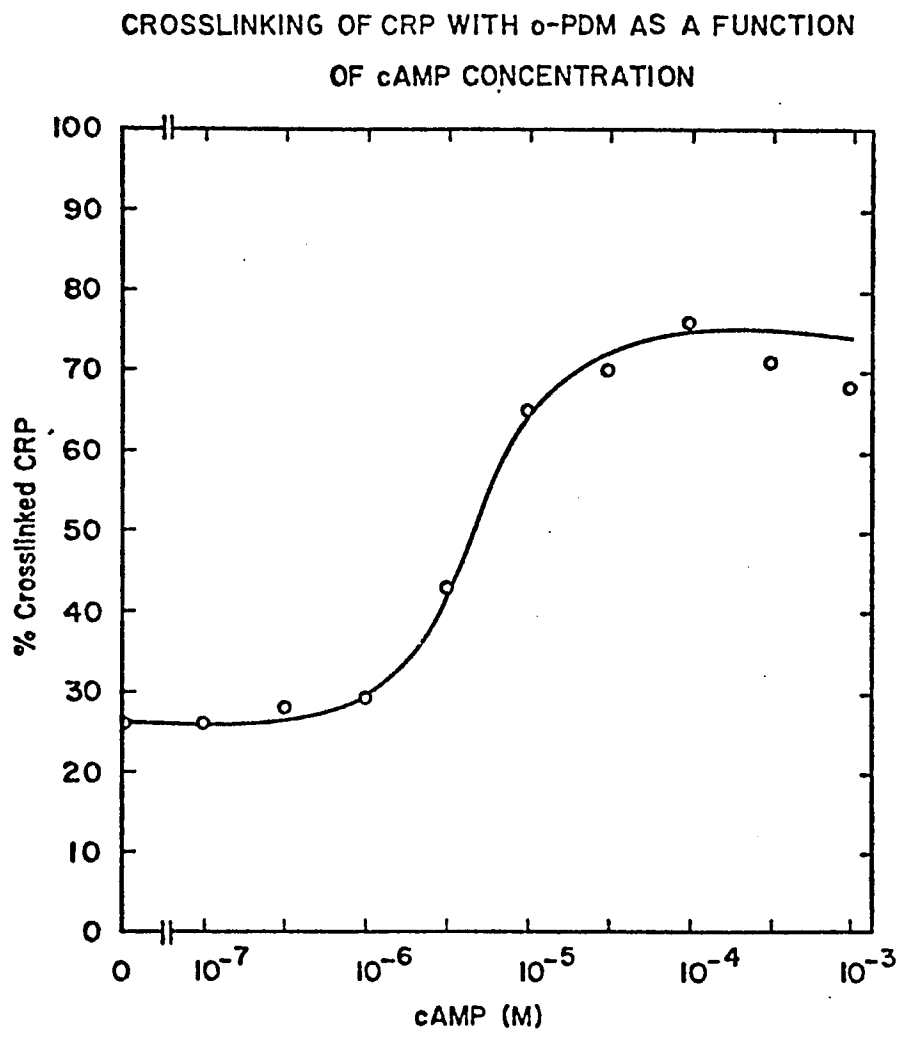

CROSSLINKING OF CRP WITH o-PDM AS A FUNCTION OF cAMP CONCENTRATION
% Crosslinked CRP
100
90
80
70
60
50
40
30
20
10
0
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
cAMP (M)

Figure 14. Effect of $d(I\text{-}C)_n$ and $d(A\text{-}T)_n$ on the rate of crosslinking in the presence of cAMP. The reaction mixtures contained in 190 ul : 25 ug CRP (0.55 nmoles), 0.1 M Hepes (pH 8), 0.4 mM cAMP and 35 nmoles of $d(I\text{-}C)_n$ or $d(A\text{-}T)_n$ where indicated. Prior to the addition of 6 nmoles oPDM, to give a final volume of 250 ul, reaction mixtures were preincubated for 5 minutes at 37°C. At indicated time intervals, 50 ul aliquots were removed and 5 ul of 0.5 M DTT was added to stop the reaction. The preparation and resolution of samples by SDS-polyacrylamide gel electrophoresis on 10 % slab gels were performed as described in Figure 9. CRP crosslinked in the presence of cAMP, (Δ——Δ); plus $d(I\text{-}C)_n$, (o---o); plus $d(A\text{-}T)_n$, (•——•).

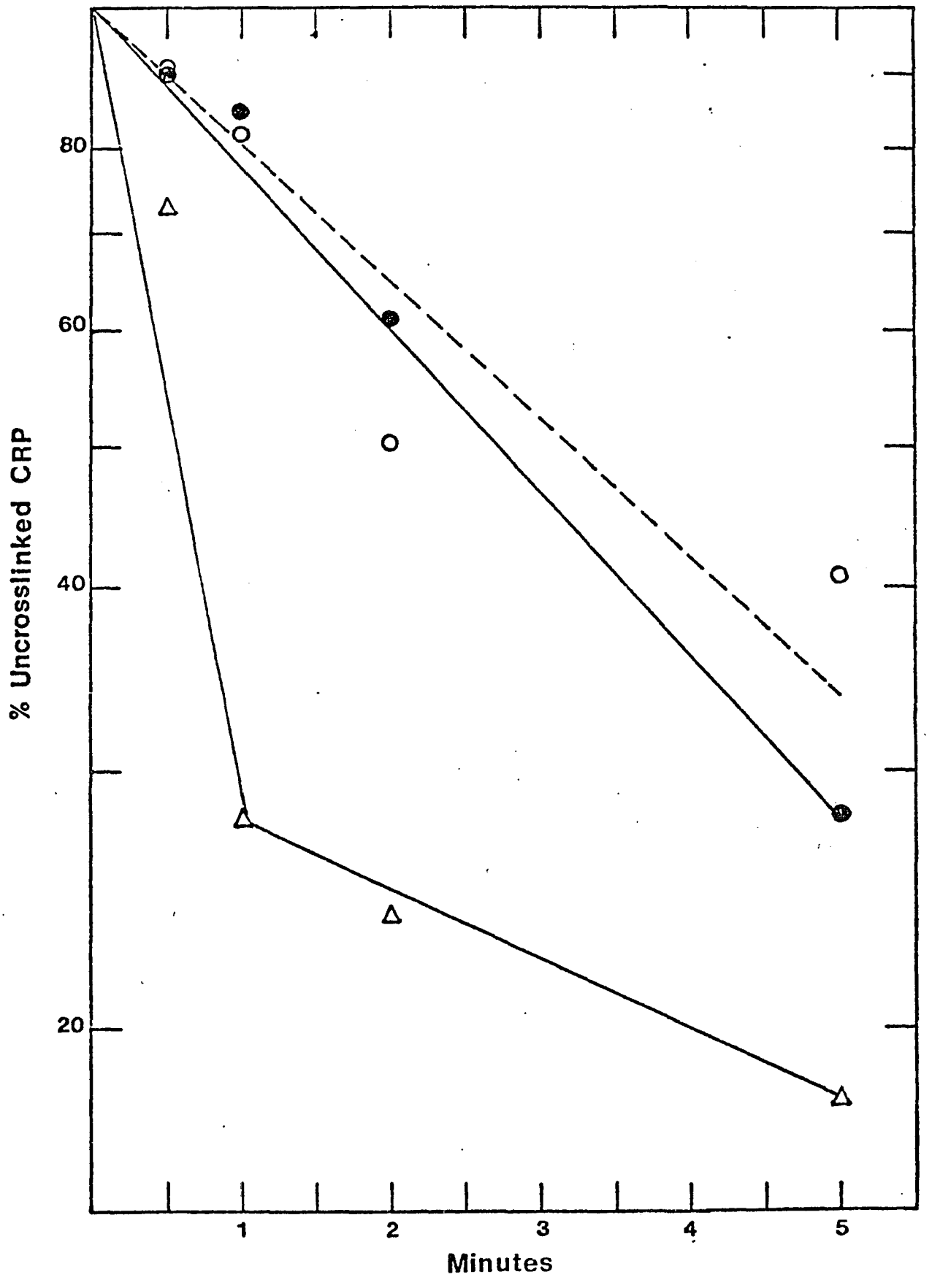

% Uncrosslinked CRP
80
60
40
20
1
2
3
4
5
Minutes

Figure 15. Time course of α chymotrypsin digestion of CRP crosslinked with oPDM in the absence of cAMP. Crosslinked CRP was prepared as described under "Materials and Methods". Proteolysis reaction mixtures contained (final volume 0.35ml) 50 mM Tris-HCl (pH 8), 87.5 ug unmodified or crosslinked CRP and 1.75 ug chymotrypsin and 1 mM cAMP where indicated. Incubations were at 30°C. At indicated time intervals, 50 ul aliquots were removed and PMSF added to a final concentration of 0.1 mM. The preparation and resolution of these samples by SDS-polyacrylamide gel electrophoresis on 12 % slab gels were performed as described in Figure 9. The first lane shows molecular weight standards: bovine serum albumin, 67,000; ovalbumin, 46,000; CRP, 22,500; sperm whale myoglobin, 17,800; CRP, 12,500. The last lane shows unmodified CRP incubated with chymotrypsin and 1 mM cAMP for 60 minutes.

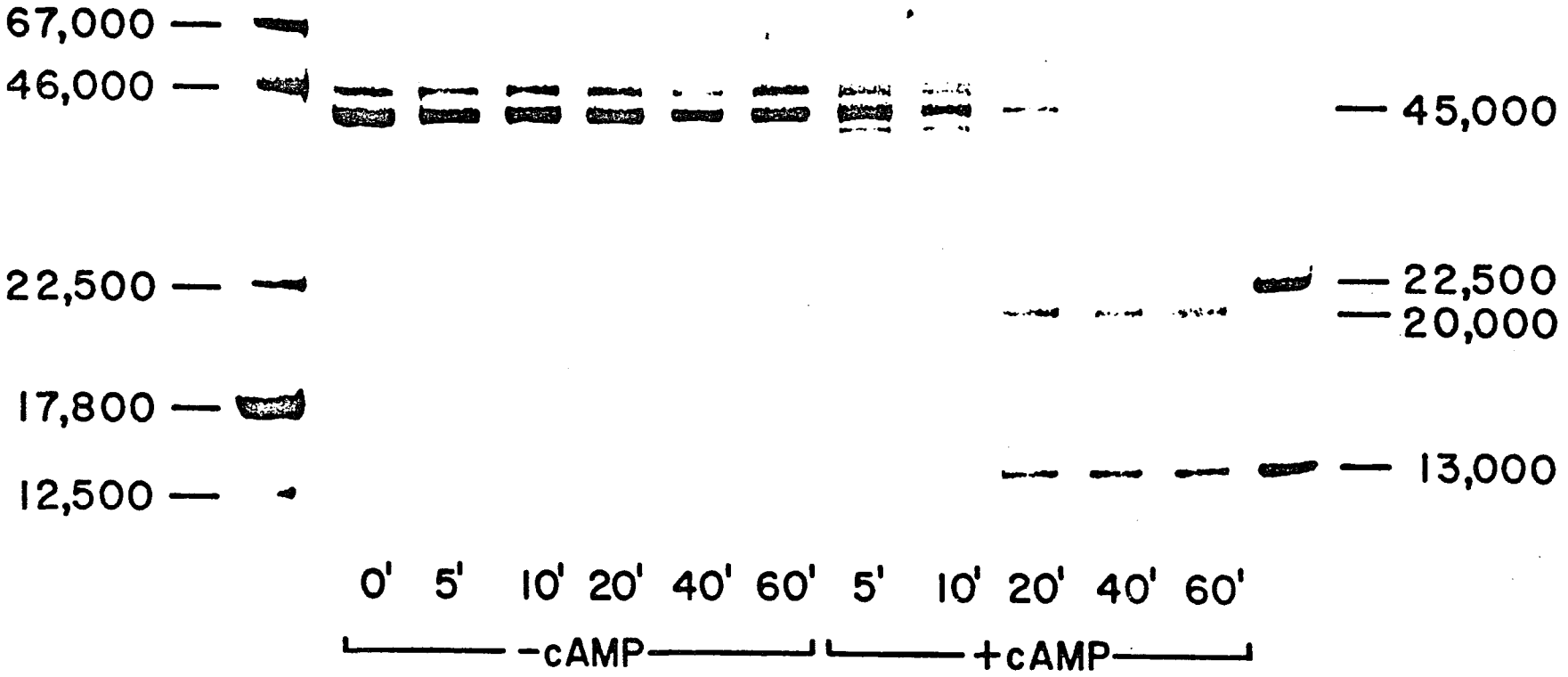

67,000
46,000
22,500
17,800
12,500
45,000
22,500
20,000
13,000
0' 5' 10' 20' 40' 60' 5' 10' 20' 40' 60'
-cAMP
+cAMP

Figure 16. Time course of α chymotrypsin digestion of CRP crosslinked with oPDM in the presence of cAMP. The experiment was performed as described in Figure 15 except that the last lane shows unmodified CRP incubated with only chymotrypsin for 60 minutes.

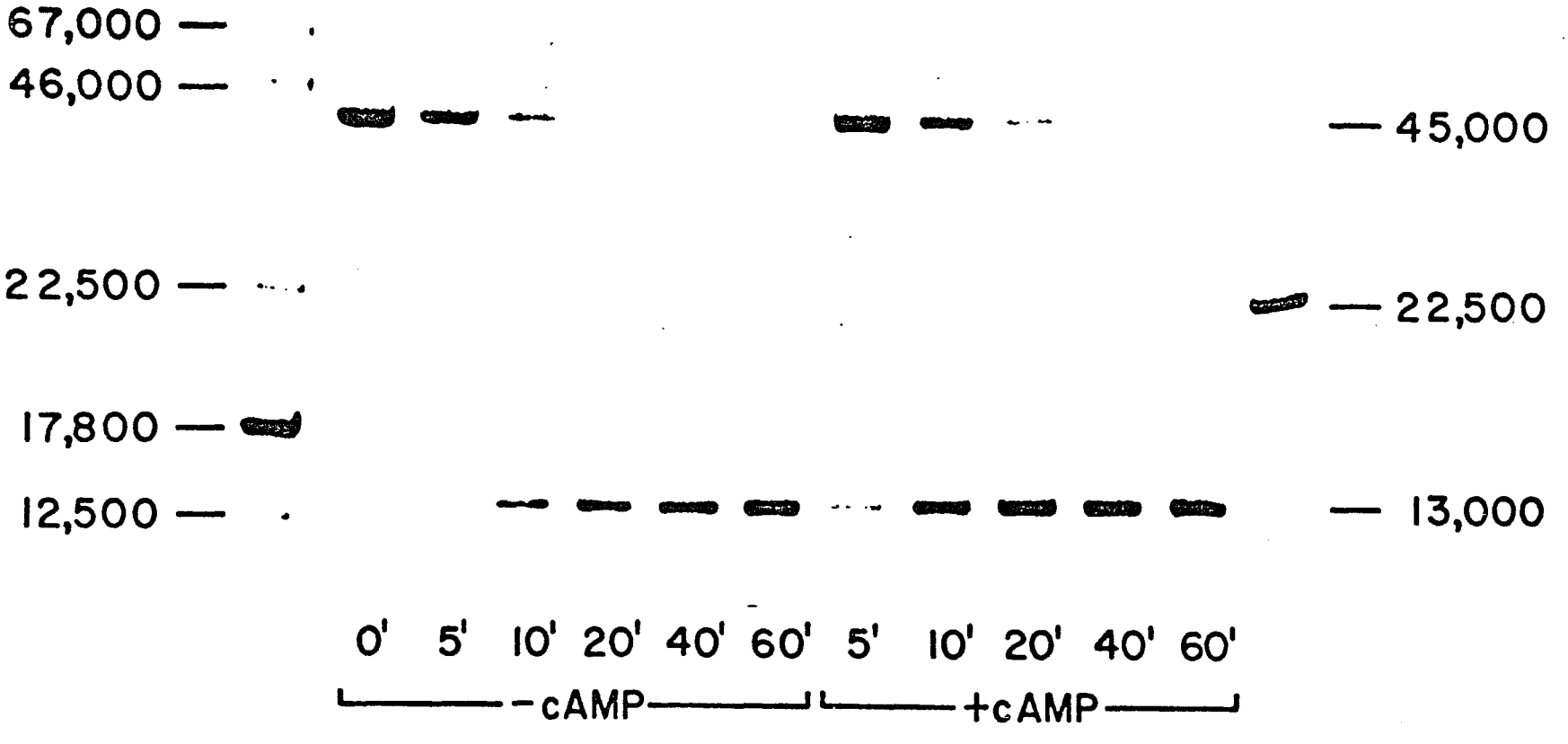

67,000
46,000
22,500
17,800
12,500
45,000
22,500
13,000
0' 5' 10' 20' 40' 60' 5' 10' 20' 40' 60'
-cAMP
+cAMP

Figure 17. Effect of cAMP on the digestion of crosslinked CRP by α chymotrypsin, trypsin and subtilisin. Crosslinked CRP was prepared as described under "Materials and Methods". Proteolysis reaction mixtures contained (final volume 50 ul): 13 ug crosslinked CRP, 50 mM Tris-HCl (pH 8), 0.25 ug chymotrypsin, trypsin or subtilisin and 1 mM cAMP where indicated. After incubation at 37°C for 15 minutes, PMSF was added to a final concentration of 0.1 mM. The preparation and resolution of samples by SDS-polyacrylamide gel electrophoresis on 12 % slab gels were performed as described in Figure 9. CRP crosslinked in the absence of cAMP, (A); CRP crosslinked in the presence of cAMP, (B).

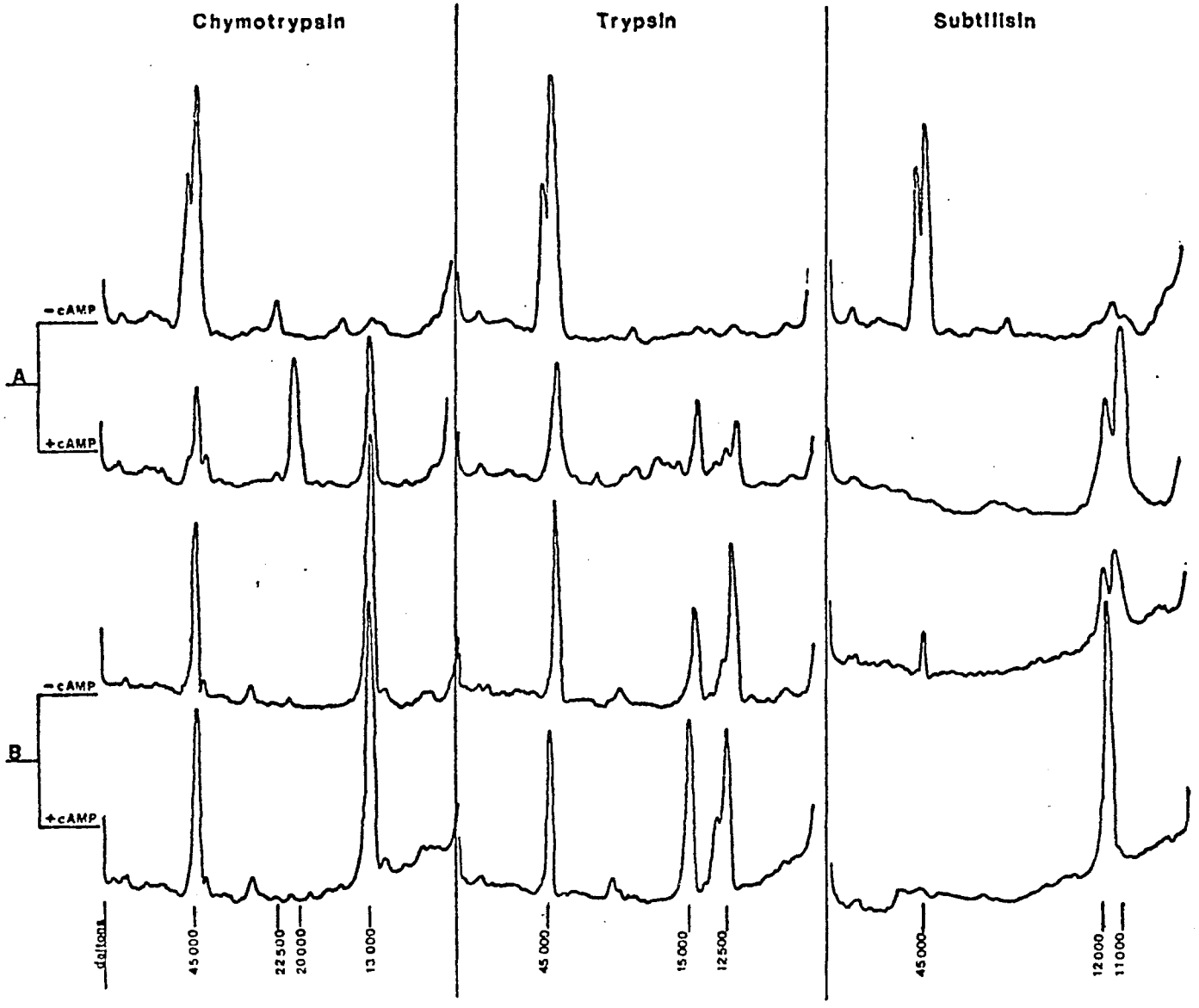
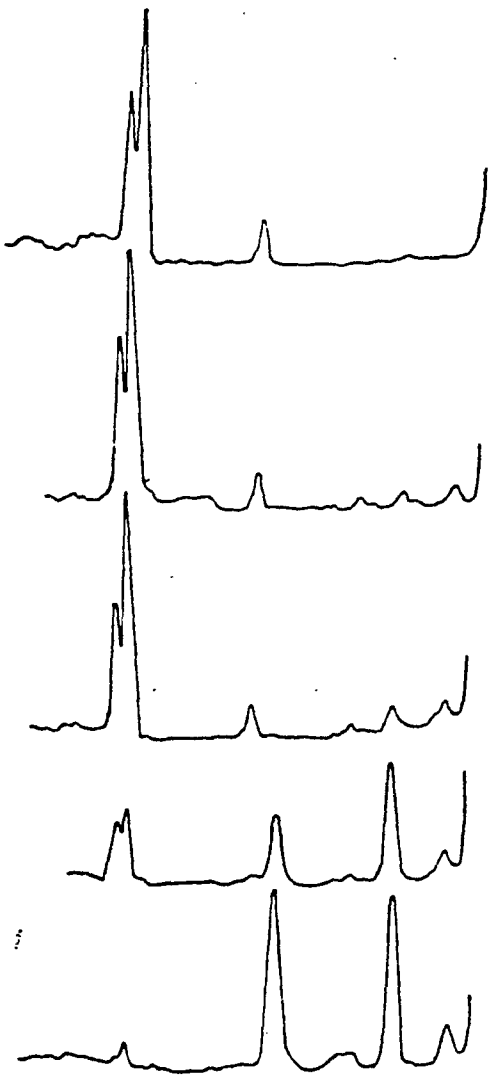

Chymotrypsin
Trypsin
Subtilisin
A
B
−cAMP
+cAMP
−cAMP
+cAMP
daltons
45 000
22 500
20 000
13 000
45 000
15 000
12 500
45 000
12 000
11 000

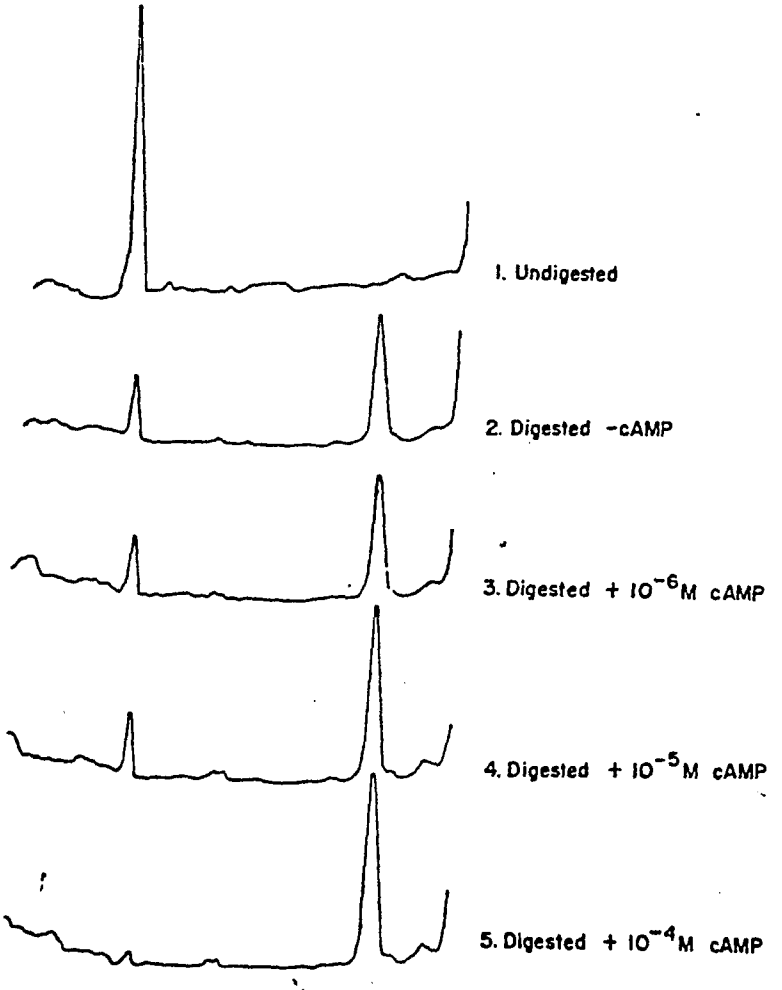
Figure 18 A & B. The effect of cAMP on chymotrypsin digestion of CRP crosslinked with oPDM in the absence (A) and presence (B) of cAMP. Crosslinked CRP was prepared as described under "Materials and Methods". Proteolysis reaction mixtures contained (final volume 50 ul) 50 mM Tris-HCl (pH 8), 12.5 ug CRP, 0.5 ug chymotrypsin and the indicated concentrations of cAMP. After incubation of samples for 30 minutes at 30°C, proteolysis was terminated by addition of PMSF to a final concentration of 0.1 mM. Preparation and resolution of samples on 12 % slab gels were performed as described in Figure 9.

A. CHYMOTRYPSIN DIGESTION OF CRP CROSSLINKED IN THE ABSENCE OF cAMP

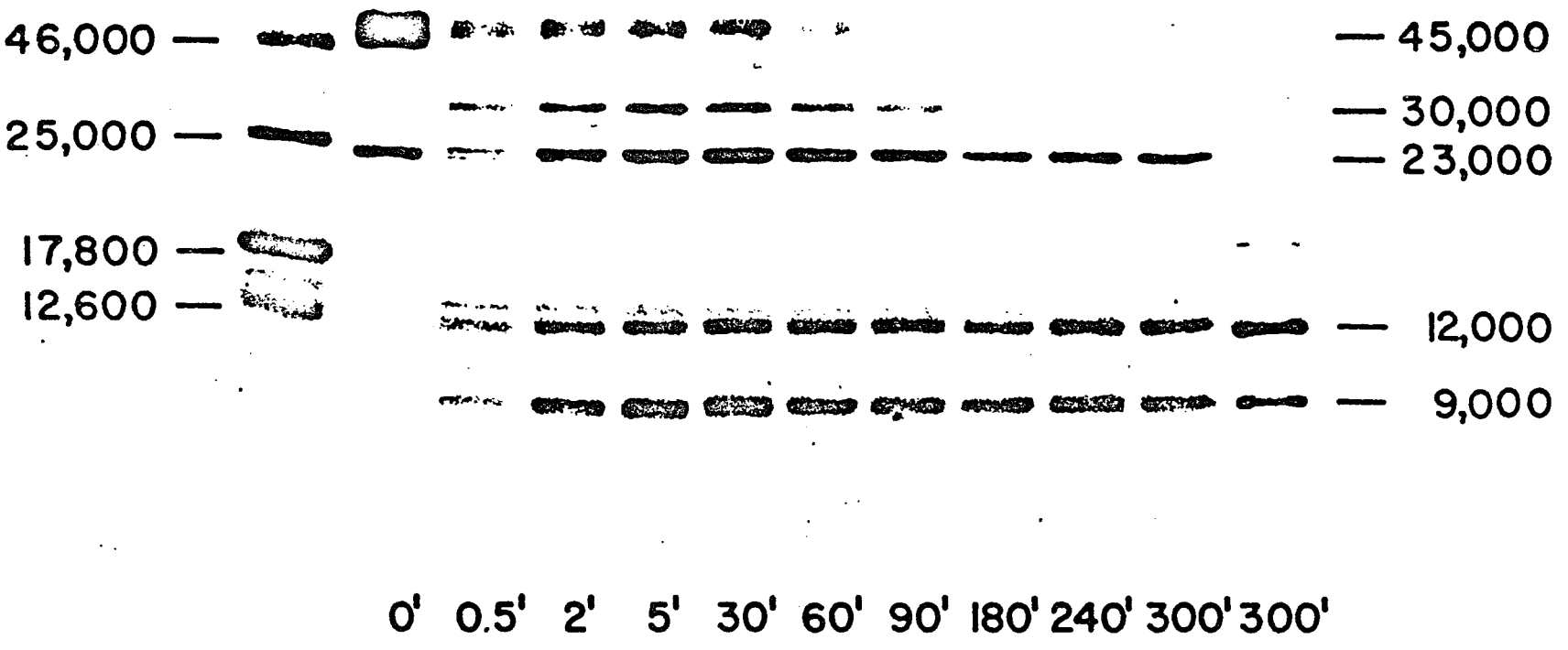


1. Undigested
2. Digested −cAMP
3. Digested + 10^{-6}M cAMP
4. Digested + 10^{-5}M cAMP
5. Digested + 10^{-4}M cAMP

B. CHYMOTRYPSIN DIGESTION OF CRP CROSSLINKED IN THE PRESENCE OF cAMP



Figures 19. and 20. SDS-chymotrypsin digestion of CRP crosslinked with oPDM in the absence (Fig. 19) and presence (Fig. 20) of cAMP. Crosslinked CRP was prepared as described under "Materials and Methods". Predigestion mixtures contained 324 ug crosslinked or native CRP, 0.125 M Tris-HCl (pH 6.8), 1 mM EDTA, 1 mM DTT, 10% glycerol and 0.5% SDS in a final volume of 1.2 ml. The mixtures were heated in a boiling water bath for 5 minutes. After cooling, 24 ug of α chymotrypsin was added. Incubation was at 37°C. At indicated time intervals, 20 ul and 100 ul aliquots were removed for protein determination and gel electrophoresis respectively. Proteolysis was stopped in the 100 ul samples by heating in a boiling water bath for 2 minutes. Aliquots of 40 ul were directly applied to a 15% slab gel with a 4.2% spacer. Protein determinations and electrophoresis were performed as described under "Materials and Methods". Molecular weight standards are shown in the first lane: ovalbumin, 46,000; chymotrypsinogen, 25,000; sperm whale myoglobin, 17,800; horse heart cytochrome c, 12,600.


46,000
25,000
17,800
12,600
45,000
30,000
23,000
12,000
9,000
0'
0.5'
2'
5'
30'
60'
90'
180'
240'
300'
300'

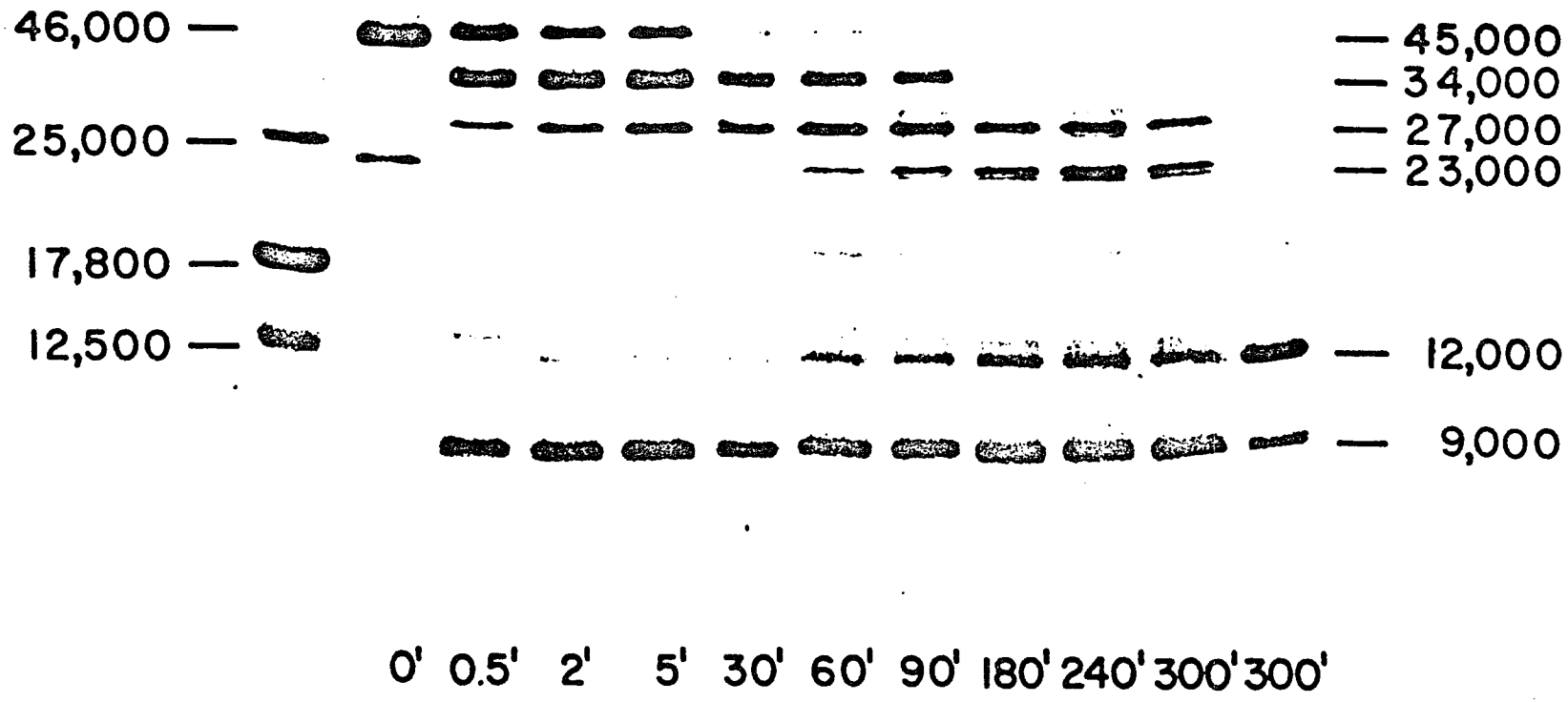
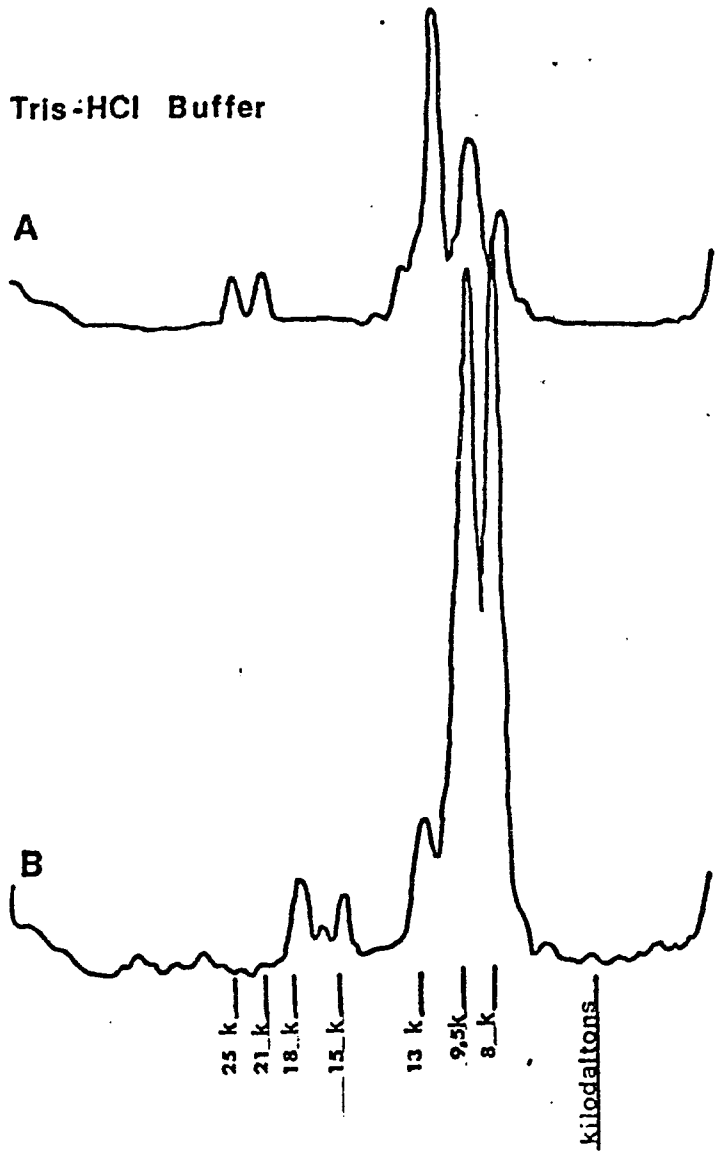

46,000
25,000
17,800
12,500
45,000
34,000
27,000
23,000
12,000
9,000
0'
0.5'
2'
5'
30'
60'
90'
180'
240'
300'
300'

Figure 21. Proteolysis of crosslinked CRP with Staph. aureus V8 protease in an SDS-Tris-HCl or SDS-NH_4HCO_3 buffer system. Predigestion mixtures contained (final volume 0.1 ml): native or crosslinked CRP, 30 ug prepared as described under "Materials and Methods", either 0.125 M Tris-HCl (pH 6.8) or 0.125 M NH_4HCO_3 (pH 8), 1 mM EDTA, 1 mM DTT, 10% glycerol and 0.5% SDS. Samples were heated in a boiling water bath for 5 minutes. After cooling, 5 ug of SAP was added. Incubation was at 37°C. At 60 minutes, proteolysis was terminated by incubation in a boiling water bath for 2 minutes. Aliquots of 40 ul were directly applied to a 15% SDS-polyacrylamide slab gel with a 4.2% spacer. Electrophoresis and densitometry was performed as described under "Materials and Methods".

A. Digestion of CRP crosslinked in the absence of cAMP.

B. Digestion of CRP crosslinked in the presence of cAMP.


Tris-HCl Buffer
A
B
25 k
21 k
18 k
15 k
13 k
9,5k
8 k
kilodaltons

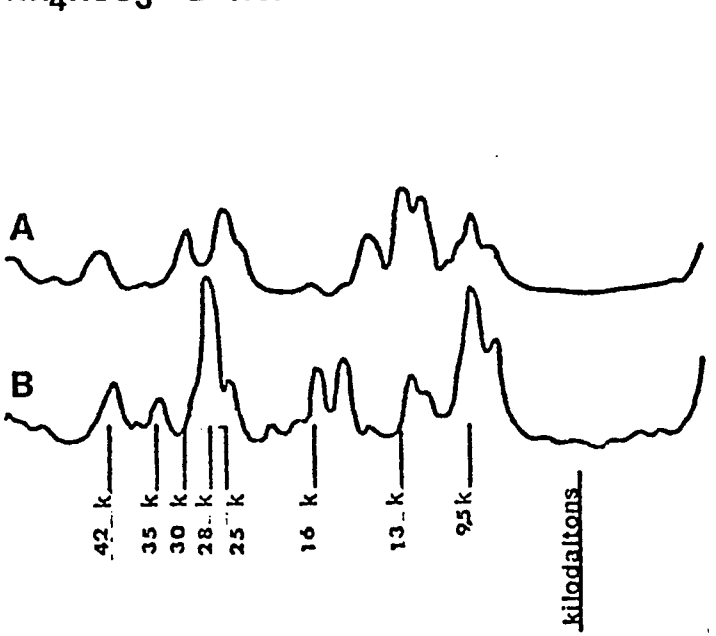

NH4HCO3 Buffer
A
B
42 k
35 k
30 k
28 k
25 k
16 k
13 k
9,5k
kilodaltons

Figure 22. Chymotrypsin digestion of MBS crosslinked CRP. MBS crosslinked CRP was prepared as described under "Materials and Methods". Digestion mixtures contained (final volume 50 ul): 12 ug crosslinked CRP, 50 mM Tris-HCl (pH 8), 1 mM cAMP where indicated and 0.25 ug chymotrypsin. After incubation at 37°C for 40 minutes, PMSF was added to a final concentration of 0.1mM. The preparation and resolution of samples by SDS-polyacrylamide gel electrophoresis on 15% slab gels with a 4.2% spacer were performed as described in Figure 9.

A) MBS crosslinked CRP minus chymotrypsin.

B) MBS crosslinked CRP digested in the absence of cAMP.

C) MBS crosslinked CRP digested in the presence of cAMP

D) oPDM crosslinked CRP digested in the presence of cAMP.

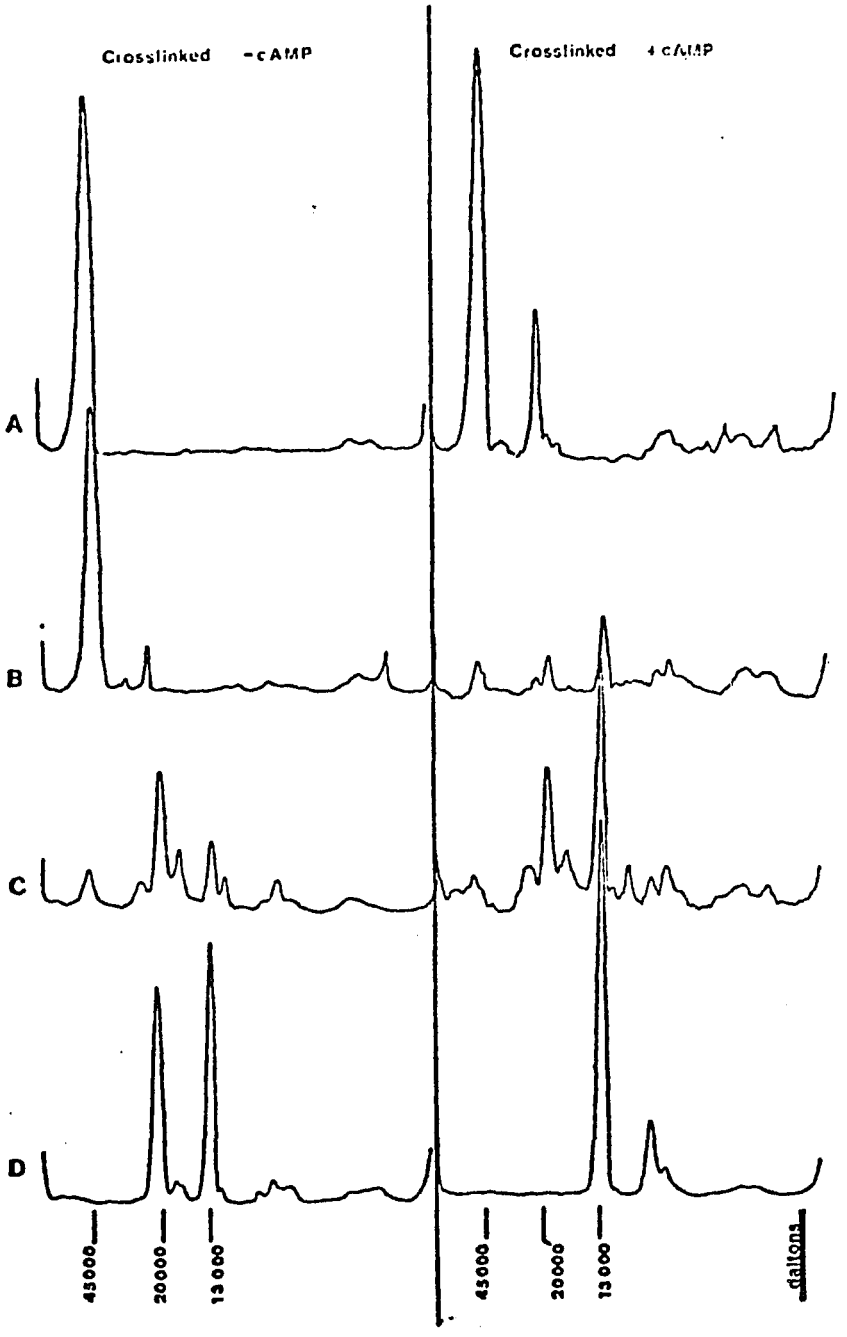

Crosslinked -cAMP
Crosslinked +cAMP
A
B
C
D
45000
20000
13 000
45000
20000
13 000
daltons

Figure 23. SDS-chymotrypsin digestion of MBS crosslinked CRP. MBS-crosslinked CRP was prepared as described under "Materials and Methods". Predigestion mixtures contained 28 ug crosslinked CRP, 0.125 M Tris-HCl (pH 6.8), 1 mM EDTA, 1 mM DTT, 10% glycerol and 0.5% SDS in 95 ul. The mixtures were heated in a boiling water bath for 2 minutes. After, cooling, 5 ug of chymotrypsin was added (final volume 100 ul). After incubation at 37°C for 60 minutes, proteolysis was terminated by heating in a boiling water bath for 2 minutes. Aliquots (40 ul) were directly applied to a 15% SDS-polyacrylamide slab gel with a 4.2% spacer. Electrophoresis and densitometry were performed as described under "Materials and Methods".

Digestion of: A) CRP crosslinked in the absence of cAMP.
B) CRP crosslinked in the presence of cAMP.
C) CRP crosslinked with oPDM in the absence of cAMP.

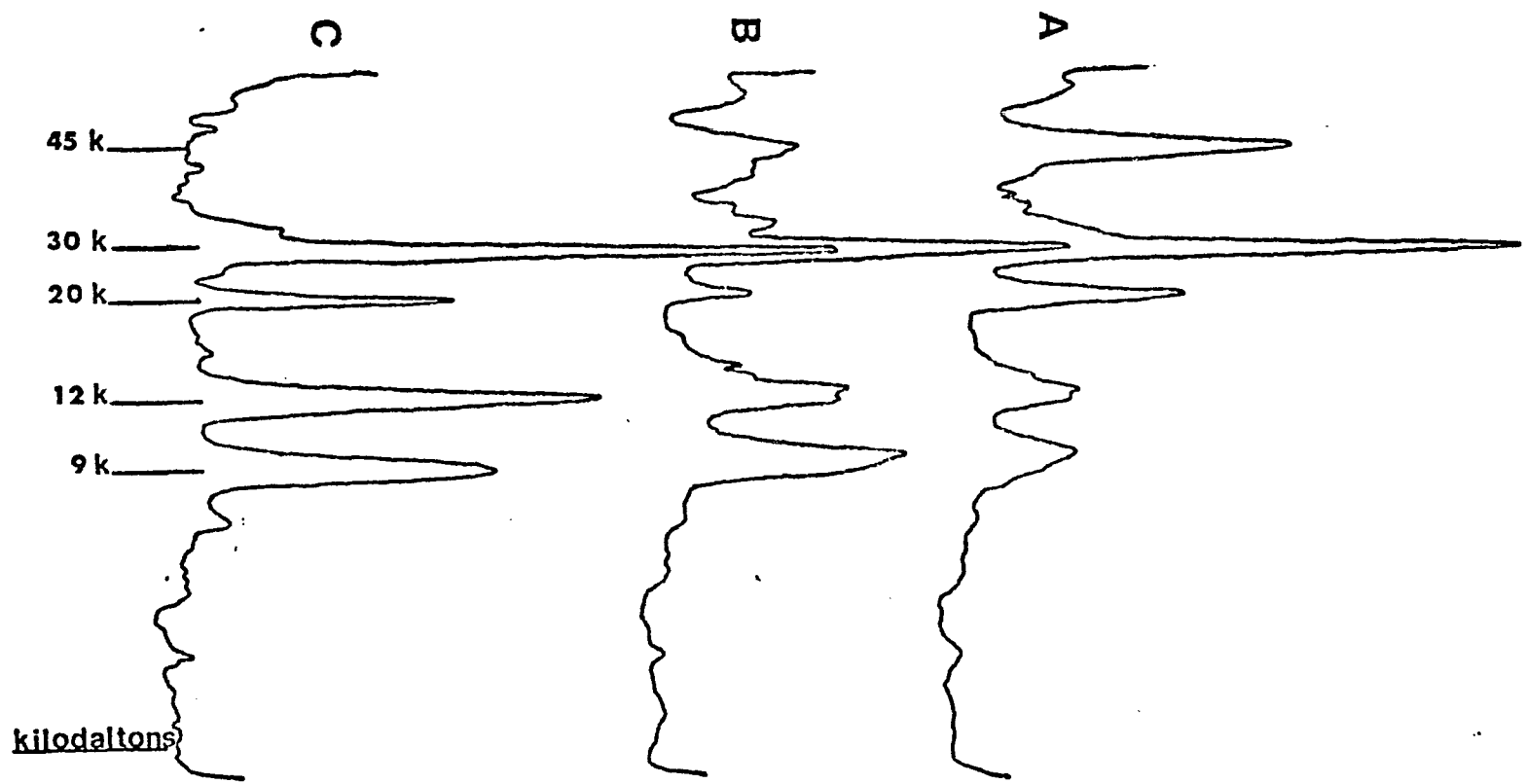

C
B
A
45 k
30 k
20 k
12 k
9 k
kilodaltons

Figure 24. Absorption spectrum of TNBS modified CRP. TNBS modified CRP was prepared as described under "Materials and Methods". The spectrum of 0.3 mg/ml TNP-CRP in 50 mM potassium phosphate buffer (pH 7), 0.1 M NaCl, and 0.1 mM EDTA (dialysis buffer) was determined with a Beckman Acta III recording spectrophotometer with a reference cell containing control CRP which had been carried through the same treatment except that TNBS was omitted. Curve A, TNP-CRP; Curve B, TNBS (50 mM) in dialysis buffer.

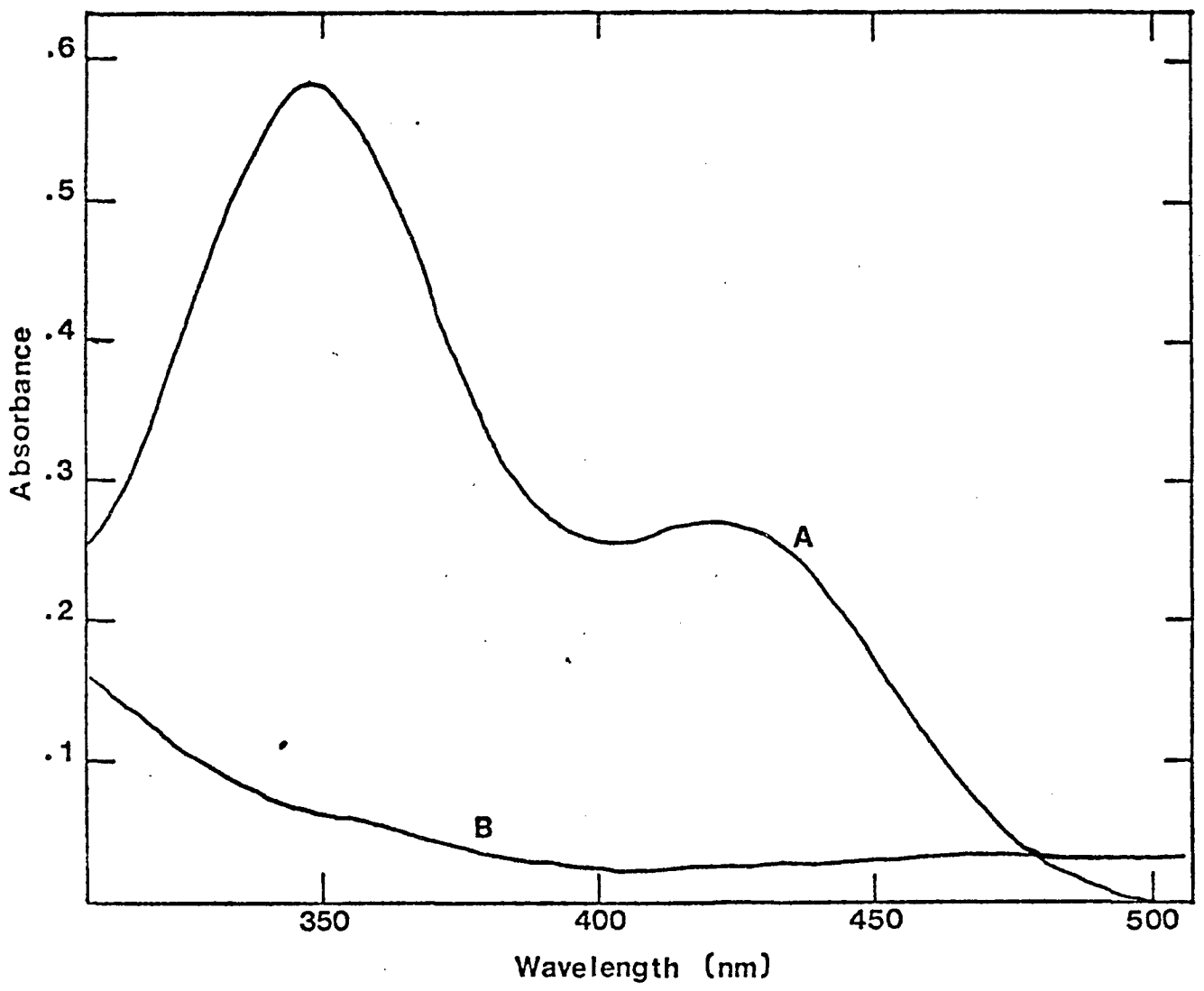

.6
.5
.4
.3
.2
.1
Absorbance
A
B
350
400
450
500
Wavelength (nm)

Figure 25 A & B. $(^3H)d(A-T)_n$ (A) and $(^3H)d(I-C)_n$ (B) binding activity of TNP-CRP. Assay mixtures contained (final volume 0.25 ml): 40 mM Hepes (pH 8), 3.5 nmoles $(^3H)d(A-T)_n$ (980 cpm/nmole) or $(^3H)d(I-C)_n$ (890 cpm/nmole), the indicated amount of protein sample minus cAMP (o) or plus 0.4 mM cAMP (•). Reactions were carried out as described under "Materials and Methods". Solid lines, unmodified CRP; broken lines, TNP-CRP.

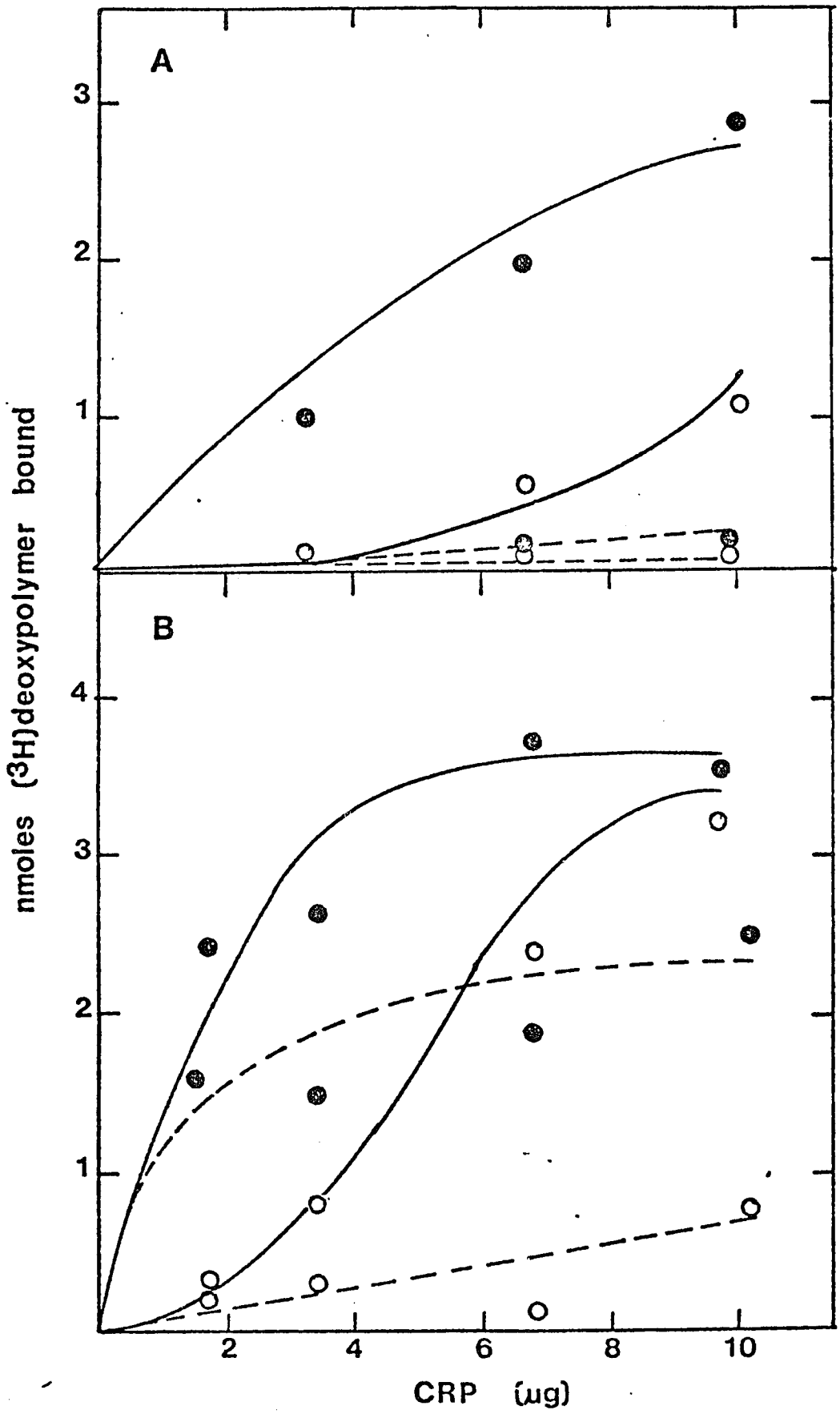

A
B
3
2
1
4
3
2
1
2
4
6
8
10
nmoles (3H)deoxypolymer bound
CRP (ug)

Figure 26. Fragments of CRP and cores.

FRAGMENTS DERIVED FROM CRP AND CORES

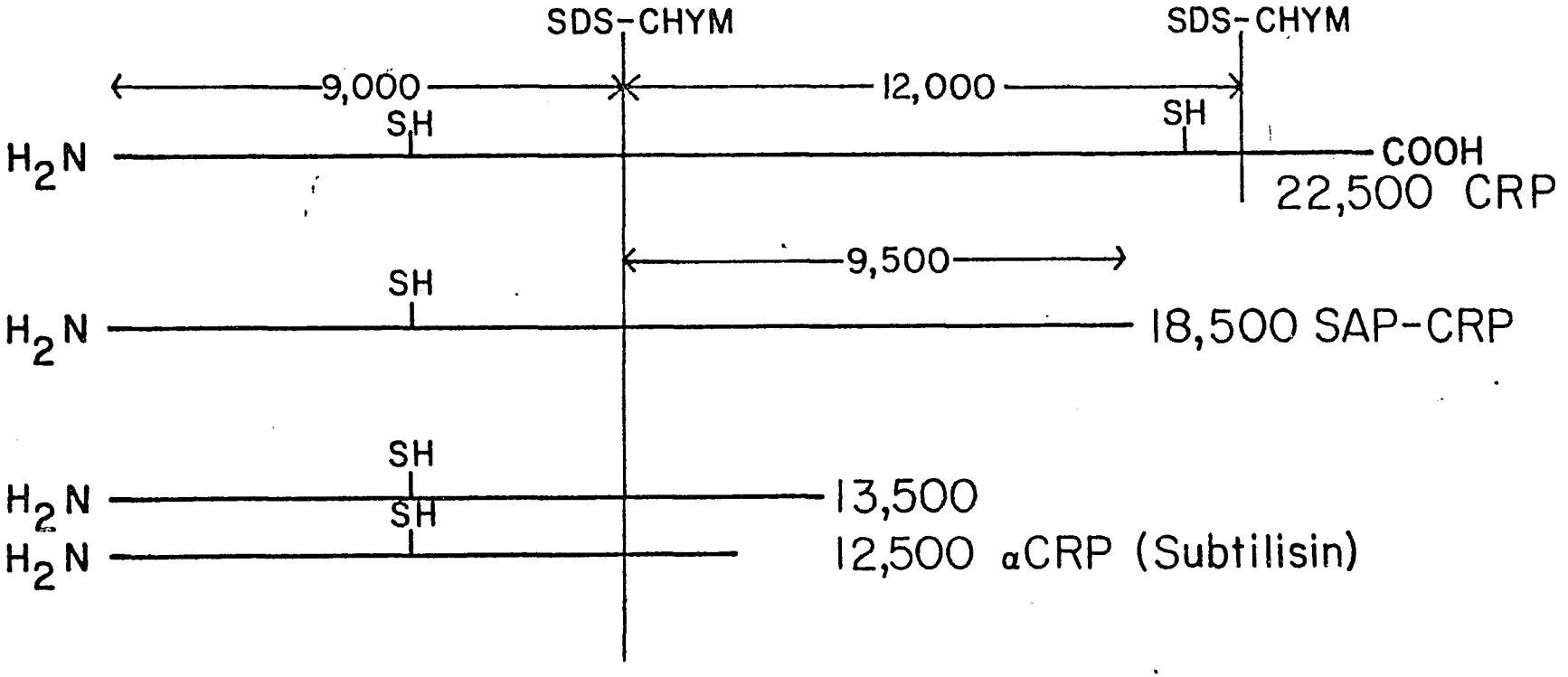


TABLE I. DTNB TITRATION OF CRP AND SAP-CRP SULFHYDRYL GROUPS.

NUMBER OF SH GROUPS	CRP	SAP-CRP CORE
Native conditions	2.3	0
Denaturing conditions (0.4% SDS)	4.1	2-3

The reaction was carried out at 24°C in a 0.5 ml quartz cuvette containing 0.1 M sodium phosphate buffer (pH 8), 0.1 M NaCl, 100 ug of protein, 8×10^{-5} M DTNB and 0.4% SDS where indicated. The absorbance at 412 nm was continuously recorded against a blank containing buffer, NaCl, DTNB and SDS where indicated. The absorbance at the reaction plateau (15 minutes) was used to calculate the number of titrated SH groups (ε= 13,600 $M^{-1}cm^{-1}$; Ellman, 1959). The molecular weight of CRP and SAP-CRP used to calculate the number of SH groups per mole of protein was 45,000 and 36,000 respectively.

TABLE II. (^{14}C)NEM MODIFICATION

	Native Conditions	Denaturing Conditions
	cpm	cpm
Undigested CRP	1958	4458
CRP Fragments Molecular Weight		
22,500	83	110
12,000	1398	2377
9,000	346	2053

Reaction mixtures contained (final volume 0.5 ml): 0.5 mg CRP, 20 mM sodium phosphate buffer (pH 8),and 0.4mM (^{14}C)NEM (specific activity 13,000 cpm/nmole). Reactions under denaturing conditions also contained 0.5% SDS. Incubations were at 37°C for 40 minutes. Reactions were terminated by addition of DTT to a final concentration of 40 mM. Samples were dialyzed overnight at room temperature vs 2 L of 10 mM sodium phosphate (pH 7) and 0.1 M NaCl. Chymotrypsin digestion of (^{14}C)NEM labeled CRP was performed as described in Figures 19 and 20. Electrophoresis on 15% SDS-polyacrylamide slab gels was as previously described except that the gels were minimally stained and destained to allow detection of protein bands. The radioactivity in the protein bands was determined using the technique of Goodman and Matzura (1971). Bands were excised and allowed

to air dry overnight in scintillation vials. Protein was eluted by layering 0.2 ml of a 99:1 (v/v) solution of 30% H_2O_2 and concentrated ammonium hydroxide over the bands and incubating at 37°C overnight. A 10 ml solution of ethylene glycol monomethyl ether-Liquifluor (3:5 parts) was added to the dissolved bands and the samples were counted. The counting efficiency was 30%.

TABLE III. EFFECT of cAMP on the $t_{1/2}$ of the CROSSLINKING REACTION with oPDM and pPDM.

ratio of PDM to CRP	cAMP	$t_{1/2}$ (minutes) oPDM	pPDM
	-	15	50
1:1	+	7	8
	-	4.3	6
10:1	+	0.7	1

The $t_{1/2}$ values were determined as described in Figure 12 except that either 25 nmoles or 2.5 nmoles of oPDM or pPDM were added where indicated.

TABLE IV. THE EFFECT of CYCLIC NUCLEOTIDES on the CROSSLINKING of CRP by oPDM.

Nucleotide added	M	% crosslinking
none	-	36
5'-AMP	10^{-3}	31
cGMP	10^{-3}	35
cAMP	10^{-5}	24
	10^{-4}	55
	10^{-3}	85
cTuMP	10^{-5}	34
	10^{-4}	83
	10^{-3}	80

Reaction mixtures contained (final volume 50 ul) 5 ug CRP (0.11 nmoles), 50 mM Hepes (pH 8), 0.11 nmoles oPDM and indicated concentrations of nucleotides. After incubation for 10 minutes at 37°C, reactions were terminated by addition of ME to a final concentration of 40 mM. The preparation and resolution of samples by SDS-polyacrylamide gel electrophoresis on 10% slab gels were performed as described in Figure 9.

TABLE V. Effect of oPDM on CRP. Samples were prepared as described under "Materials and Methods". The cAMP binding dissociation constant and number of binding sites per CRP protomer were determined by Scatchard analysis (Fig. A-3), described under "Materials and Methods". Results show the standard deviation for measurements from four sets of experimental data. DNA binding assays were performed as described under "Materials and Methods".

TABLE V. EFFECT of oPDM on the cAMP RECEPTOR PROTEIN

	Untreated	Treated -cAMP	Treated +cAMP
% Crosslinking	---	95	100
cAMP Binding			
Kd	$1.6\pm0.2 \times 10^{-5}$M	$4.4\pm0.6 \times 10^{-5}$M	$1.3\pm0.5 \times 10^{-5}$M
Number of binding sites	1.1 ± 0.2	1.5 ± 0.1	1.2 ± 0.1
$\frac{(^3H)d(I\text{-}C)_n \text{ Binding}}{(\text{nmoles})}$			
-cAMP	0.15	0.03	0.10
+cAMP	1.27	0.05	0.33

TABLE VI. Effect of oPDM on CRP pretreated with DTNB. Reaction mixtures contained (final volume 0.3 ml) 200 ug (4.4 nmoles) CRP, 0.1 M Hepes (pH 8) and 10 nmoles DTNB. After incubation for 20 minutes at 37°C one sample received 0.1 mM cAMP and 40 nmoles of oPDM was added to both treated samples; another sample was untreated with respect to oPDM modification. Final reaction volumes were 0.5 ml. Reactions proceeded as described under "Materials and Methods". Assays for (^{3}H)cAMP binding and (^{3}H)d(I-C)$_n$ binding activity were performed as described under "Materials and Methods".

TABLE VI. EFFECT of oPDM on the cAMP RECEPTOR PROTEIN PRETREATED with DTNB

	Untreated	oPDM Treated (-cAMP)	oPDM Treated (+cAMP)
% Crosslinking	0	0	0
Specific Activity (pmoles (^{3}H)cAMP/mg CRP	8951	6431	9130
(^{3}H)d(I-C)$_n$ Binding (nmoles)			
-cAMP	0.16	0.14	0.20
+cAMP	1.18	0.95	0.98

Figure A-1. A. Nucleotide sequence of the operator-promoter region of the lac operon (from Pastan and Adhya, 1976). B. Nucleotide sequences around the CRP binding sites in lac and gal. Palindromes are underlined and brackets mark homologous sequences (from Musso et al., 1977a).

A

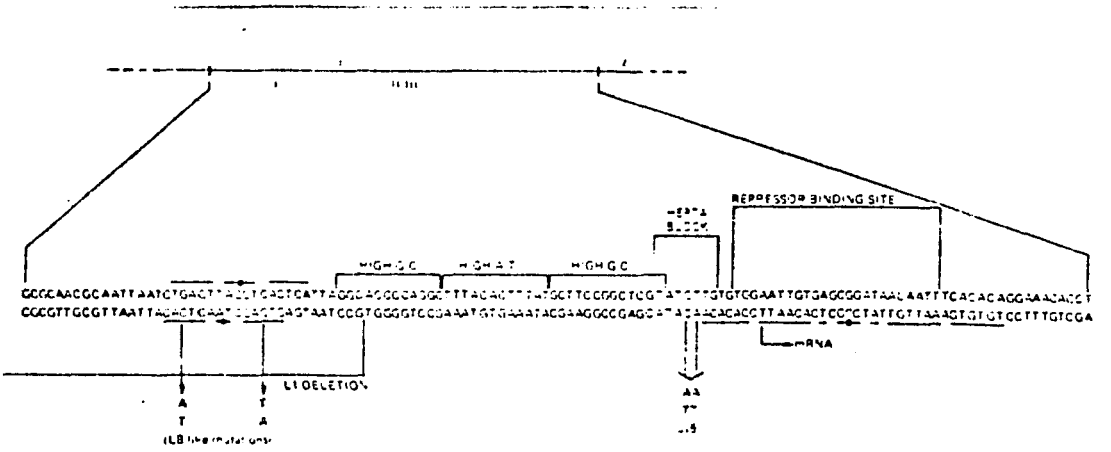


FIG. 2. *Genetic map and nucleotide sequence of the promoter-operator region of the lac operon (27, 35, 43). The promoter locus, p, located between the lac repressor gene, i, and the operator, o, is subdivided into sites I and II·III. Site I is the cyclic AMP and CRP recognition element and carries two overlapping partial twofold symmetry elements indicated by horizontal lines. Point mutations, like L8, which affect cyclic AMP and CRP recognition, always show change of either of two G-C nucleotide pairs located in symmetrical positions in one of the symmetry elements into an A-T pair (W. Reznikoff, personal communication). This suggests that the cyclic AMP·CRP complex "sees" this DNA symmetry. Promoter site II·III contains the A-T-rich block, spanned by two G-C-rich blocks, as well as the heptanucleotide block, the possible implications of which are discussed in the text. The mutation, UV5, to cyclic AMP and CRP-independent promoter affects the hepta block. The operator locus, the repressor-binding site, also contains a partial twofold symmetry, indicated by horizontal broken lines. The horizontal arrow indicates the start of lac transcription.*

B

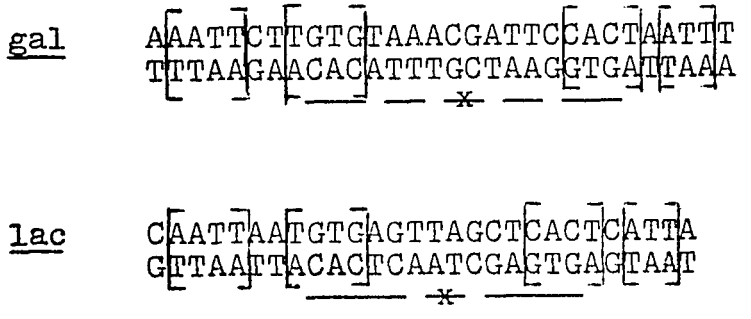
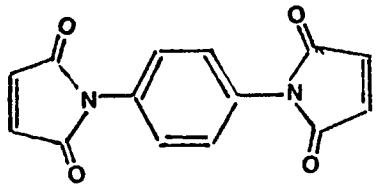


Figure A-2. Structures of bifunctional reagents.

A. N.N'-(1,4-phenylene)bismaleimide (pPDM), (Wold,1967).

B. meta-maleimido-benzolyl-N-hydroxysuccinimide (MBS), (Kitagawa and Aikawa, 1976).

A)



B)

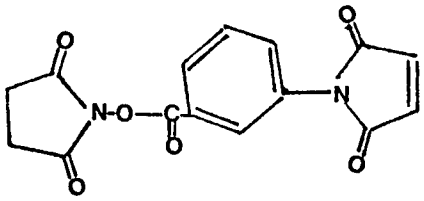


Figure A-3. Scatchard plots of cAMP binding to CRP and oPDM crosslinked CRP. Equilibrium binding studies were performed as described under "Materials and Methods". The data was plotted according to Scatchard (1949) where r equals the molar ratio of cAMP bound per protomer and L is the nucleotide molarity. The slope and x intercept were calculated from the least-squares line to determine the dissociation constant and number of cAMP binding sites respectively.

(o---o),CRP crosslinked in the absence of cAMP;

(●---●),CRP crosslinked in the presence of cAMP ;

(Δ---Δ),unmodified CRP.

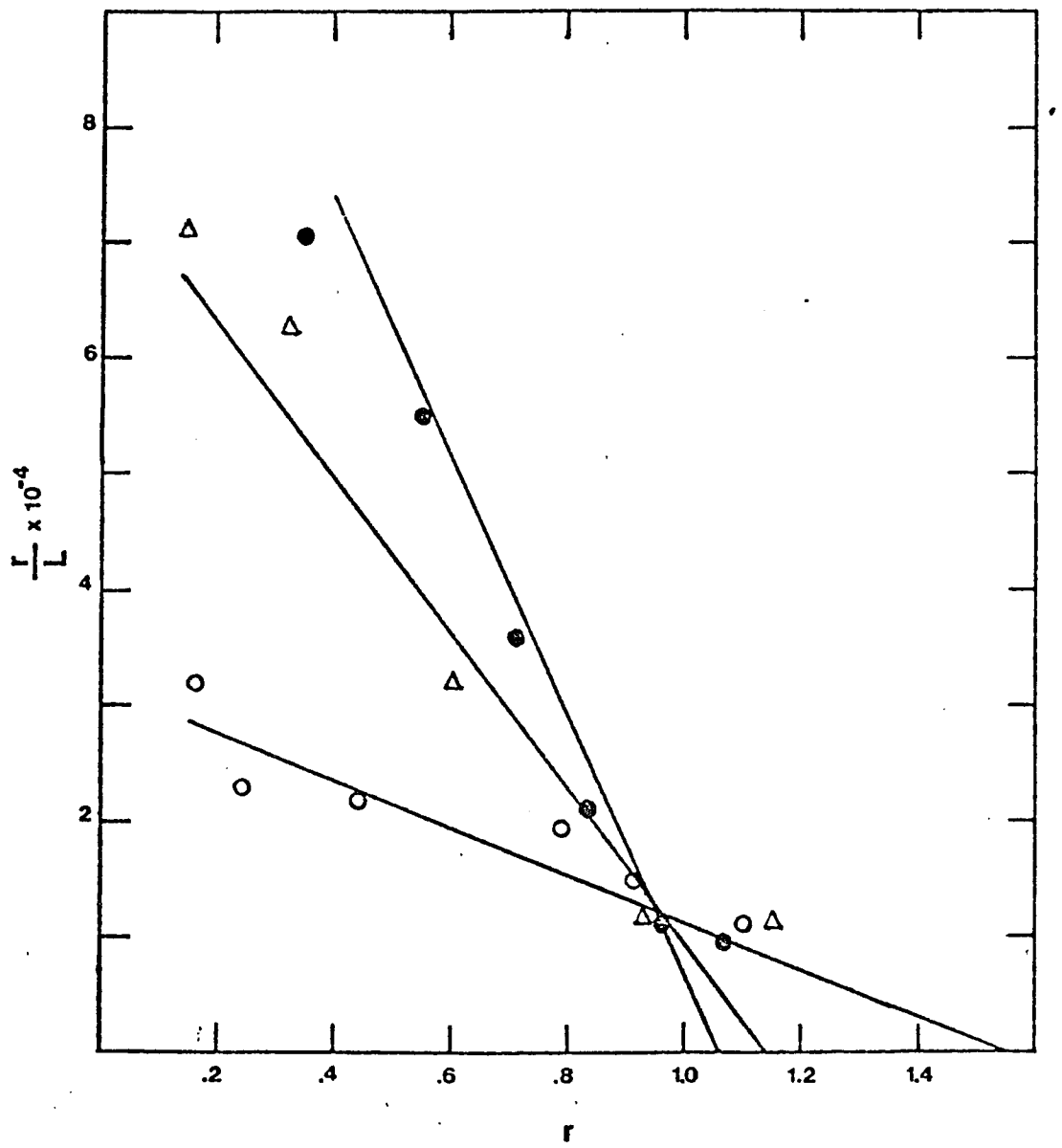

8
6
4
2
$\frac{r}{L} \times 10^{-4}$
.2
.4
.6
.8
1.0
1.2
1.4
r

TABLE A-I. PURIFICATION OF CRP

Stage of Purification	Volume (ml)	Protein (mg)	Activity (units)	Specific Activity (units/mg)	Yield (%)	A_{280}/A_{260}
Lysate Supernatant	1800	41,400	270,000	6.5	-	0.50
Polyethyleneimine supernatant	1300	22,100	163,000	7.4	-	0.72
Bio Rex 70, I	200	260	352,000	1354	100	0.93
Sephacryl S-200	225	90	276,400	3070	79	1.12
DNA cellulose	110	35.5	220,000	6300	63	1.70
Bio Rex 70, II	6.5	32.5	210,000	6460	60	1.78

Data is from Eilen et al. (1978)

TABLE A-II. PROPERTIES OF CRP, αCRP[1] and SAP-CRP.

	CRP	αCRP	SAP-CRP
Subunit M Wt.	22,500	12,500	18,000
Protomer M. Wt.	45,000	25,000	36,000[2]
Total SH	4	2	2-3
"Buried" SH	2	2	2-3
Relative Net Positive Charge	+++	++	+
Binds cAMP	yes	yes	yes
Binds DNA, pH 8.0 +cAMP	yes	no	no

[1]data from Eilen et al. (1978)

[2]recently established by Sephadex G-75 gel filtration (Hiroji Aiba, personal communication).

TABLE A-III. SUMMARY OF THE EFFECTS OF CHEMICAL MODIFICATION ON CRP.

Reagent	Possible groups modified	Effect on cAMP Binding	Effect on DNA Binding	Additional Effects
5-5'-dithiobis(2-nitro-benzoic acid) (a)	SH	none	inhibits	mediates disulfide cross-linking of intraprotomer subunits + cAMP
phenylenedimaleimides	SH, NH_2	none[b]	inhibits	crosslinks intraprotomer subunits ± cAMP
meta-maleimido-benzolyl-N-hydroxysuccinimide	SH + NH_2	none[b]	inhibits	crosslinks intraprotomer subunits ± cAMP
N-(3-pyrene)maleimide	SH	none[b]	inhibits	bifunctional reagent which does not crosslink CRP.
dimethylsuberimidate	NH_2	none	none	bifunctional reagent which does not crosslink CRP.
trinitrobenzenesulfonic acid	NH_2	none[b]	inhibits	limited modification allows CRP to differentially bind deoxypolymers

(a) Eilen and Krakow (1977b)

(b) Little effect under conditions where greater than 50% DNA binding function is lost.